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# *Immuno-Catalysis*





# Immuno-Catalysis

And Related Fields of Bacteriology and Biochemistry

(Second Edition, Revised and Enlarged)

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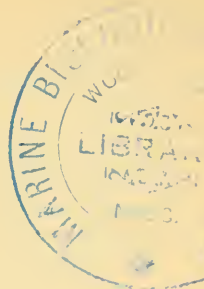
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## *Preface*

CULTIVATION of the special fields of science at first tends to express the attributes of the particular subjects of investigation, the immediate practical objectives and the technical instrumentalities which prove serviceable in each field. Specialization of investigational method and equipment, of vocabulary and of the body of accumulating fact and conclusion may become extreme, resulting in an unfortunate degree of isolation of the field and its workers. With growing insight, however, the special lore of the particular field is gradually seen to represent special aspects of the same truths which may be perceived also from other fields, to exemplify laws of more general validity. Isolationism in science is then a phase of narrow understanding and immaturity. Integration enriches the special fields in technique and content, and broadens the horizons of the workers.

The studies of Pasteur on the alcoholic, and tartaric, butyric and acetic acid fermentations brought about by particular micro-organisms were certainly among the principal original sources of scientific insight into the specific causation of infectious diseases. Interrelationships between the phenomena of immunity and of biocatalysis, also, have been perceived by men of discernment working in both fields, Ehrlich, Morgenroth, Zinsser, Landsteiner, Avery, Marrack, Todd and Northrop, to mention but a few. Ehrlich, indeed, borrowed his famous lock and key simile expressing the specificity of antibody with respect to antigen from Emil Fischer's studies on enzyme specificity. As a matter of practice, however, physiological chemists working with enzymes have only rarely concerned themselves with infectious disease and immunity, and bacteriologists and immunologists have paid too little heed to the many developments in enzymology paralleling developments in their own fields. The fullness of the integration possible between the fields of enzyme chemistry, immuno-chemistry and the mechanisms of infectious disease, has, indeed, in the writer's belief,

been indicated for the first time in this volume by M. G. Sevag, *Immuno-Catalysis*.

In Sevag's treatment of immuno-catalysis we discern that enzyme, substrate, and specifically inhibitive reaction products, have their respective counterparts in antigen, the antibody precursors, and specific antibodies. This is no mere analogy, for antigens in truth do determine the specificity of newly forming antibodies by a catalytic mechanism; many protein enzymes have been proved to be antigenic; the chemical configurations upon which specificity depends in enzyme and immune reactions are often analogous or identical.

Specialists either in enzymology or immunology will find the first three sections of the book a reservoir of experimental fact, considered with insight and woven together in a remarkable synthesis.

The enzymes of plants which are pharmacologically active, the enzymes of snake venoms and the enzymes of pathogenic bacteria are considered in detail with reference to their chemical activities *in vitro*, their pharmacodynamic actions *in vivo*, and with respect to specific immunity against them.

Consideration of this array of data, which is little known to most students of immunity and of infectious disease, leads the reader to the conclusion that many diverse symptoms and lesions of infectious disease may find their explanation in terms of the action of biocatalysts of the parasite which can alter vital substrates of the host. Much of the pathological symptomatology of bacterial and viral disease may thus ultimately come to be understood as special manifestations of enzyme action. Chemical and pharmacological studies with the lecithinases, proteases and nucleases of snake venoms and of those associated with pathogenic Clostridia, and studies of hyaluronidase and fibrinolysin afford striking pertinent cases.

Success in research and success in teaching are in no small part dependent upon discernment of the interrelationships of things; the deeper the insight into phenomena which seem superficially unrelated, the more clearly they may often be perceived to rest upon more fundamental, general laws of matter and energy. Teachers and investigators dealing with biochemistry, with bacteriology and immunity and with the mechanisms of infectious disease will, I believe, find in this informative book an expanded horizon and a challenge to further investigations.

In the five years between the appearance of the first and the present edition of *Immuno-Catalysis* the intimate relationships between biocatalysis and innumerable phenomena in the medical sciences have become even more clearly apparent. The subject is maturing. The author's treatment of the subject of *Immuno-Catalysis* has likewise matured. It is our sincere belief that the present volume will prove stimulating and helpful to the many biochemically trained investigators and teachers who are reinvigorating the medical sciences.

Stuart Mudd, M.D.

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## AUTHOR'S COMMENTS AND ACKNOWLEDGMENTS

THE excellent reception accorded to the first edition of this book was highly gratifying and stimulating. This necessitated the preparation of a second edition, work on which started in early 1946. The contents of the book have been enlarged by including many new subjects of biochemical, enzymological and immunological interest, including a chapter on the physiology and biochemistry of anaphylaxis.

The author takes this occasion to express his indebtedness to many friends, readers and reviewers here and abroad whose comments have been a constant source of stimulation.

- During the preparation of the manuscript for this edition I have enjoyed the privilege of carrying on research under grants to the University of Pennsylvania from the Josiah Macy, Jr., Foundation and from the United States Public Health Service.

I wish to express my appreciation to Dr. Otto Rosenthal of the Department of Research Surgery, and to Dr. Seymour S. Kety of the Department of Pharmacology of the Graduate School of Medicine, and to Dr. John R. Preer of the Department of Zoology, for valuable aid received in the preparation of certain sections. Special thanks are due to Dr. John Flick and Professor Stuart Mudd for their constant stimulating interest and their painstaking reading of the entire manuscript. Their most valuable suggestions are incorporated into the text.

M. G. Sevag







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# *Immuno-Catalysis*



## Introduction

IT IS A universal phenomenon of nature that every living form must struggle to perpetuate its existence. In this pursuit its primary energy is spent in the search for food. Its chemical activities determine the extent the food can be made use of. From the standpoint of a free living bacterium, it is immaterial whether it derives its food from dead matter, or within a suitable host; the host is simply another medium for its propagation. The disease, death, and immunity, in case of recovery, caused by its multiplication in a host, are incidental events. Only extremely parasitic living forms are dependent on a host. The focus of our interest must of necessity be the nature and the intensity of the chemical activities of micro-organisms, for these constitute their biologically indispensable characteristics and determine the nature and the fatality of the diseases they cause.

Micro-organisms of varying degrees of virulence, such as Group A  $\beta$ -hemolytic streptococci, *Streptococcus viridans*, *pneumococcus*, *Salmonella aertrycke*, *C. diphtheriae*, *Shigella dysenteriae*, *Cl. tetani*, *Cl. botulinum*, *Cl. welchii*, *Staphylococcus aureus*, *B. proteus*, members of the colon group, etc., produce their exotoxins and endotoxins *in vitro* under special or routine laboratory growth conditions. It is immaterial how these toxins are produced; whether they are products of bacterial secretion, autolysis, or a result of the activities of certain specific bacterial enzymes on the medium, or are extracted from the intact cell by chemical manipulation, has no bearing on the basic aspect of the question. As these poisons can be elaborated by the bacteria outside of, or in the body of hosts, they are the products of their specific physiological activities in the course of their normal growth and death.

Many of the pathogenic bacteria produce acute exotoxic diseases. However, in chronic infections, the disease agents either do not elaborate such fatal poisons, or they are rendered less harmful by the host. Whatever may be the answer, at the end a serious condition may develop to a greater or lesser degree as the result of the physiological

activities of the disease agents. In this connection it may be noted that infections by *Spirochaeta pallida*, *M. tuberculosis*, the leprosy bacillus, *Actinomyces* and malaria parasites etc., are diseases which habitually run a very slow course and do not show evidence of any fatal toxin which can overwhelm the host before the defenses can be mobilized. Cutaneous and mucous eruptions, inflammatory discharges, the swelling of the regional nodes, the malaise, the fever and sensation of chilliness, numerous disturbances in the normal physiology of the infected host may likewise be looked upon as the consequences of the interaction between specific bacterial components and the environment offered by the hosts.

These considerations bring us to the examination of the possible physiological rôle of the various constituents of bacteria. Hence the morphological and cultural characteristics, and biochemical activities of bacteria must be studied from the point of view of their relation to infectious diseases, as well as from the view of the biology of the bacterium *per se*.

Examining the facts from the standpoint of the biology of bacteria, we see that the enzyme activities of bacteria are indispensable for their growth and reproduction *in vitro*, as well as *in vivo*. Invasiveness and fatalness of a bacterial infection are conditioned generally not only by the degree of the resistance offered by the host but, more specifically, by the degree and nature of the enzyme activities of invasive bacteria. The antigenic property of a bacterium, on the other hand, is a consequential property; it is conditioned by its propagation in a host for a requisite length of time. This property of bacterial substances would never have come to light if the bacterium had followed an entirely free-living, non-parasitic existence. These facts indicate no doubt that with the exception of highly parasitic forms micro-organisms are not dependent on a host, and, more specifically, not on the antigenic properties of their constituents for existence and propagation.

On the contrary, the antigenic property is not of utility for the bacterium, but is detrimental to its prolonged propagation within a host. For example, certain infections are followed by effective immunity which is instrumental in wiping out the bacteria from the body of a host. On the other, in chronic infections the ineffectiveness of the antigenic property of certain disease agents assures them of a prolonged existence in a host.



Despite these facts, the property which makes certain bacterial constituents antigenic must play a definite rôle in the biology of a bacterium. Of these constituents, protein is the most essential one. It is not an exaggeration if we make the statement that life could not have appeared without protein substances. The proteins of the most primitive forms of life which have appeared as single cells in the evolutionary scale of living forms must have possessed attributes responsible for the antigenic property long before the appearance of animal hosts which alone have served as the means of exposing it.

Certain bacterial protein substances, as well as those of animals and plants, would appear to be endowed with catalytic powers which are essential for the physiological functions of the living forms from which they are derived. Likewise, catalytic power, as discussed in this treatise, appears to stimulate, or is responsible for the formation of specific antibodies in animals to proteins and also to certain non-protein components. From the standpoint of the biology of bacteria these facts would seem to represent a contradiction; nevertheless it is a fact that chemical forces of bacteria (or their constituents) which accelerate their *in vitro* activities, as well as their invasive, infectious and growth activities in a host, are also instrumental in setting up in the host highly specific antagonistic immune mechanisms which block or neutralize these very activities of bacteria. The highly specific nature of these relationships, characteristic of enzyme reactions, are strong indications that the mechanism of the above mentioned bacterial activities are of catalytic nature.

The action of enzymes usually results in the formation of reaction products which specifically inhibit the action of these enzymes. Similarly, as discussed in this treatise, antigens as enzymes, or enzymes as antigens, produce antibodies, as finished reaction products, the only function of which, as far as our present knowledge goes, is to neutralize the specific biological activities of antigens.

Practically all proteins foreign to the species possess a special sort of biocatalytic power, namely that of stimulating and directing specifically the synthesis of antibody. This familiar property of antigenicity must be considered a special kind of biocatalytic power, and it is possessed by enzyme proteins, in common with other proteins. Since we are at present in the dark concerning the specific functions of the latter proteins in the living cells, it is premature to consider them devoid of

special properties characteristic of the known enzymes which catalyze anabolic and catabolic reactions. The answer to the question of whether or not antigenicity and enzyme-activity are two distinguishable or identical biocatalytic properties of proteins can come from the results of studies designed with this question in mind. The results of certain studies to be discussed in the text would seem to show that under appropriate conditions specific reactivity as enzyme and as antigen may, and frequently do in fact, reside in the same specific chemical configurations of the protein molecule. This view has brought us to consideration of the well-known analogy that exists between enzyme and immune reactions. Ehrlich, finding that toxin-antitoxin, ricin-antiricin reactions are highly specific, was the first to call attention to the similarity between immune reactions and those of enzymes observed by Emil Fischer. Many investigators, more particularly Landsteiner, have referred to this analogy on numerous occasions. The parallelism between the specificities of these two reactions has been extended to d- and l-specificity by Landsteiner and Van der Scheer, and to  $\alpha$ - and  $\beta$ -configurational specificity by Avery and Goebel and their associates. However, the question as to why the specificities of these two classes of reaction approach and nearly coincide with each other has remained until the present still very much of a riddle. The present author has critically analyzed the known facts concerning this question, and the belief has grown in him that his interpretation of the basic facts regarding the above mentioned analogy and the related questions present a useful contribution to the sciences of immunology and biochemistry. This belief has prompted him to prepare the present treatise, which he humbly offers to the reader under a new title, "Immuno-Catalysis."

## *Part I*

# Antigens as Biocatalysts

**I**N THE introductory part of this study the concept formulated as a working hypothesis attributed to antigens (bacteria, toxins and proteins) properties similar to those of biocatalysts. Among these properties the possible catalytic rôle of antigens in the formation of antibodies will be discussed more fully. A clear understanding of this aspect of antigens may yield additional information about immune processes, as well as the possible rôle the bacterial antigens might play in the biology of bacteria.

The most outstanding property of antigens is, by definition, the stimulation of the formation of antibodies. Although the reactions between antigens and their specific antibodies have been extensively studied, the rôle of antigens in the formation of antibodies has not yet been clearly defined. Certain factors and conditions contributing to the production of antibodies are likewise well known, but these scattered facts have not been evaluated and integrated. The present treatise is an attempt in this direction.

To determine the conditions which control the formation of a new chemical entity, we must first establish what class of known substances this new entity belongs to. This is achieved by a detailed study of its chemical and physical properties in reference to known substances. With respect to antibody, information concerning the site of its formation, as well as its relationship to a known class of compounds will assist us further in determining the type of reactions, or chemical influences which might be responsible for its production. A review of the literature on antigens and antibodies from this viewpoint may therefore yield valuable information.

## A. THE FORMATION AND PROPERTIES OF ANTIBODIES

With the exception of iso-antibodies (natural hemagglutinins) the tissues and the blood of a normal animal theoretically are believed to

be free of *specific* antibodies capable of reacting with bacteria, and bacterial, plant and other animal proteins. The injection of these materials into an animal creates changes resulting in the formation of antibodies. With the appearance of antibodies the serum of such an animal acquires the property of entering into specific chemical combination (agglutination, precipitation) with the bacteria or the protein used for immunization. Each species of protein thus stimulates the formation of an antibody reactive *specifically* with itself.

Several investigators have stated that in parallel with the appearance of antibodies there is an increase in the serum globulin, not all of which could be accounted for by the amount of antibody present. The findings of a few investigators will be cited here. It is found (Liu, Chow and Lee, 1937) that at most two-thirds, usually under one-half, of the increase of globulin in rabbit's serum, or immunization with pneumococci is accounted for by antibody. In contrast, the examination (Bjørneboe, 1939) of 56 anti-pneumococcal rabbit sera of different types for total and specific nitrogen led to the conclusion that the increase in serum protein during immunization was due to antibody-protein. The production of antibody-protein was therefore stated to be an extra production of proteins. Immunization of rabbits with different combinations of proteins—crystalline ovalbumin, *Limulus* hemocyanin, *Viviparus* (snail) hemocyanin, crystalline edestin and Bence-Jones protein—was followed with respect to the increase in serum globulin (Boyd and Bernard, 1937). The sera were fractionated with 13.5 and 17.4 per cent sodium sulfate. The results showed that the greatest increase takes place in the 13.5 per cent sodium sulfate fraction, rising in one rabbit by 1275 per cent. The increase in the 17.4 per cent fraction, though still relatively enormous, was in general not so large as that of the 13.5 per cent fraction. The measurements of antibody showed that the great increase of globulin was not all, or even chiefly attributable to the specific antibody produced. As a rule the antibody increase was much less than that of the globulin, although the two were generally parallel. There was no antibody in the albumin fraction. These and numerous other investigations have thus shown that in response to the stimulus of a foreign protein, the animal system experiences an increase in antibody protein and at times in "non-specific" proteins (see also van der Scheer, *et al.*, 1942).

## 1. The Chemical Nature of Antibodies

Chemical tests have shown (Welsh and Chapman, 1908; Dean and Webb, 1926) that the precipitates resulting from antigen-antibody reactions contain considerably more protein than the antigen could account for. A series of quantitative analytical studies (Hartley, 1926; Felton and Bailey, 1926; Heidelberger and Kendall, 1929, 1933, 1935, 1936) have demonstrated that the precipitate obtained with protein-free pneumococcal carbohydrates and immune sera consists largely of serum globulin; 2.5 mg. of pneumococcal polysaccharide Type II precipitated 37 mg. of protein. The serum proteins isolated from these precipitates were found to be 98 per cent antibody.

The properties of diphtheria toxin-antitoxin floccules and the precipitate in other types of antigen-antibody precipitation reactions were similar to those of serum globulin (Marrack and Smith, 1930). The particulate antigens, such as bacteria and red blood corpuscles, when fully combined with antibody, moved in an electric field as though they were pure globulin (Shibley, 1926; Eagle, 1930; McCutcheon, Mudd, Strumia, and Lucké, 1930).

The effect of proteolytic enzymes, such as pepsin, trypsin and papain, on antibodies has constituted the subject of numerous investigations. These studies have shown that antibodies are destroyed rapidly by pepsin, less rapidly by trypsin. Types I and II pneumococcal antisera, and purified Types I, II, and III pneumococcal antibodies were slowly destroyed by trypsin (Felton and Kauffmann, 1927). The precipitate formed by Type I pneumococcal polysaccharide and antiserum was incubated with pepsin (pH 4.8). The destruction of precipitin ran parallel to the increase of amino nitrogen (Chow, Lee and Wu, 1937). Rosenheim (1935) found that the O-agglutinins in all serum samples from three horses immunized with *B. typhosus* were rapidly destroyed by pepsin (pH 4.6–4.8), trypsin (pH 8.6) and activated papain. The H agglutinins in serum samples obtained from each of three horses after the first immunizing course were rapidly destroyed by proteolytic enzymes. Those in samples obtained after several immunizing courses were not appreciably destroyed under identical conditions. The H agglutinins which were apparently resistant to pepsin and trypsin were not resistant to activated papain. The



globulin fractions of the serum obtained after the first and subsequent immunizing courses were hydrolyzed to approximately the same extent by proteolytic enzymes. A certain degree of resistance to pepsin by diphtheria antitoxin serum is also reported (Pappenheimer and Robinson, 1937). However, since the peptic digestions in these cases were carried out at a pH unfavorable for obtaining maximum proteolytic activity, it should not be interpreted that these immune bodies are not of protein nature. It can only mean a relative, rather than an absolute resistance to enzymes in comparison with normal serum globulins under these conditions.

*Conclusions.* Chemical analysis of purified antibody preparations and of normal globulins have failed to indicate any differences in the percentage of amino acids and total nitrogen.

Antibodies produced in rabbits to various antigens have so far been found to be indistinguishable by ultracentrifugal and electrophoretic studies from normal rabbit serum globulin. The antibodies produced in the rabbit seem to have the same isoelectric point as the corresponding  $\gamma$ -globulin derived from the normal rabbit.

How is it then that antibodies, chemically and physico-chemically identical with globulins, are serologically distinct entities? This difference has given rise to various hypotheses, which will be discussed later. It suffices to state at this point that this difference is believed to be due to certain variations in the configuration of normal globulin during its synthesis resulting in the formation of antibodies.

## B. THE ROLE OF CATALYSIS IN CHEMICAL REACTIONS AND ITS BEARING ON THE FORMATION OF ANTIBODIES

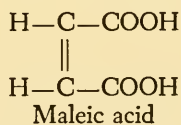
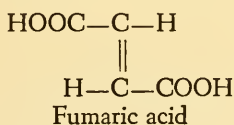
In analyzing the nature of various chemical influences which contribute to antibody formation in response to antigenic stimuli we cannot at present formulate the mechanism of the chemical reactions involved, but we can at least determine whether an antigen acts as a reactant forming a part of the final reaction product, or as a catalyst directing the synthesis of antibody globulin.

Changes in organic and inorganic substances, and changes or variations in the physiological processes of living forms are brought about by chemical influences. No matter how the mechanism of these changes or reactions are formulated they take place according to one

of the known type reactions. All chemical reactions take place according to a stoichiometrical relationship; that is, there exist numerical relations between elements or compounds in combining to form a new compound, or in tautomerizing to a new isomer, or in dissociating into elements or compounds. They do so according to definite proportions by volume and weight. Various influences, such as heat, light, or catalysts do not affect this relationship.

The influence of a catalyst does not change the stoichiometrical relationships. The catalyst does not alter the free energy of the reaction; it does not enter into any irreversibly stable union with either the reactants or the reaction products. It simply speeds up the reaction to attain the equilibrium in a shorter period of time. In many cases the reactions, in the absence of a catalyst, proceed at such a slow rate that one cannot detect their presence, but with the introduction of a suitable catalyst the reaction becomes obvious.

The structures of organic substances express mechanical and chemical meaning. All atoms or groups of atoms at special positions in the space occupied by the structure of a molecule determine the chemical and physical behavior of the whole molecule, and its groups as well. Any change in the spatial relationships of the functional groups as a whole causes a corresponding change in properties. A given empirical formula of an organic compound will often represent several isomeric compounds of the same molecular weight and elementary analysis, such as, for example, the *cis* and *trans* isomers, fumaric and maleic nature.



which have different physical and chemical properties. There are large numbers of isomers which may differ from each other only by a single physical property, namely, by the ability to rotate the plane of polarized light in a different direction. d- and l-fructose, d- and l-lactic acid, d- and l-alanine, etc., represent the class of a large number of optically active isomers. What interests us here is that some of these isomers are interconvertible under the influence of accelerating agents of catalytic nature.

In general, chemical reactions take place either (a) by the applica-

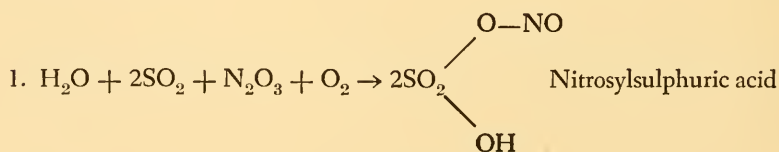


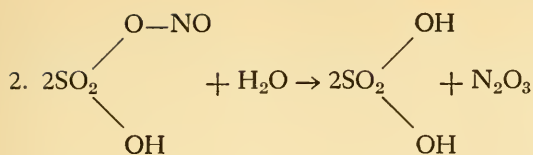
tion of heat energy; or, (b) through chemical affinities; or, (c) the application of force to non-spontaneous processes. This force may be electromotive, as in electrolytic processes; or, (d) by the accelerating effect of organic or inorganic catalysts. Two important facts are to be observed. In the first place, we note that the final products either contain the parts of the reactants, or are derived from a single substance, or a single complex molecule is formed out of two or more reactants. In contrast, the enzyme, or organic and inorganic catalysts do not enter into any irreversible union with any of the reaction products.

*The Synthesis of Sulphuric Acid by the Catalytic Contact Process.* In this process the interaction of sulphur dioxide and oxygen is the principal reaction. The interaction between the two main reactants can be hastened by the catalytic surfaces of porcelain, ferric oxide, and, more especially, by finely divided platinum spread in a fine grey powder on the fibers of asbestos to obtain maximum active surface. Under controlled temperature conditions the above gases are blown over the catalytic system forming sulphur trioxide. The platinum remains unchanged and functions continuously so long as arsenious oxide and other impurities do not poison the active surface of the catalyst.

The surface of the finely divided platinum is characterized by strong adsorbing powers. It holds the gaseous molecules with relative firmness, and it is believed that there the molecules undergo activation prior to a chemical union. Once this has taken place the active surface is set free to function again.

*The Synthesis of Sulphuric Acid in a Homogeneous Catalytic System—Chamber Process.*—Water vapor, sulphur dioxide, nitrous anhydride ( $N_2O_3$ ), and oxygen participate in the synthesis of sulphuric acid in this process. When these gases are thoroughly mixed in large leaden chambers it is assumed probably that the greater part of the acid is formed by the following two successive actions (Rideal and Taylor, 1926):





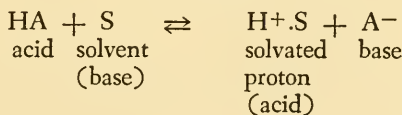
The gaseous mixture is brown; with the formation of nitrosylsulphuric acid the brown color disappears. With its decomposition into sulphuric acid and  $\text{N}_2\text{O}_3$  the color reappears. These two equations represent distinct consecutive actions and not partial equations of an interaction, for one can observe the deposition of nitrosylsulphuric acid crystals when a glass flask is used and the supply of water is deficient. The  $\text{N}_2\text{O}_3$  liberated in the second equation is immediately available to take part in the successive cycles a large number of times. In this process  $\text{N}_2\text{O}_3$  functions as a catalyst and the nitrosylsulphuric acid is the intermediate labile complex between a catalyst and the reacting substances. It corresponds to the enzyme substrate complexes generally believed to form in enzyme reactions.

## 1. Catalysis of Organic Reactions

Some of the outstanding general catalyses of organic reactions in homogeneous systems are: (a) general acid and base catalysis; (b) catalysis in aqueous concentrated acid systems; and, (c) acid catalysis in non-aqueous systems.

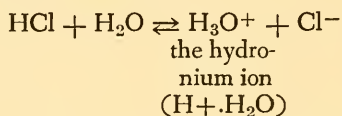
Before dealing with these catalytic processes, it will be helpful to review the modern concepts of acids and bases as well as the rôle of the solvent in processes involving acids and bases.

To extend the considerations of acids and bases beyond treatment of aqueous systems, Brönsted, Lowry, and others have defined acids as substances which yield protons ( $\text{H}^+$ ) and bases as substances which accept protons. The relative ease with which the protons are given up (or accepted) is the measure of the acid (or base) strength. The solvent itself may act as an acid or base, and, indeed, the acidity or basicity of the solvent is an important factor in the ionization processes of solutes, as shown by the following generalized reaction scheme:



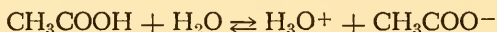
In the *solvolytic* processes, the basicity of the solvent is closely related to the acidity displayed by the acid. A system of conjugate acid-base pairs is put in equilibrium,  $A^-$  being the conjugate base of the acid  $HA$ ,  $H^+ \cdot S$  the conjugate acid of the solvent  $S$ .

A striking example of the efficacy of this theory is found in the consideration of aqueous hydrochloric acid solutions. The substance  $HCl$  is not itself an electrolyte. In the pure state its bond is strongly covalent and it is a poor conductor of electricity. The theory states that its acidity and electrolytic behavior in water solution is due to a *protolytic* reaction with the solvent:

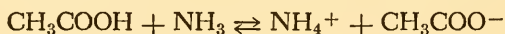


The hydrochloric acid, even up to rather high concentrations, appears to be completely ionized because of this process. This is simply due to the fact that the chloride ion is a weak base compared to the  $H_2O$  molecule, so that the equilibrium is shifted rather completely to the right.

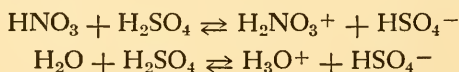
In the case of a typical weak acid, such as acetic acid, the ionization is far less complete



for the reason that the conjugate base, the acetate ion, is a rather strong base. Nevertheless, in a more basic solvent, such as liquid ammonia, this same acid is effectively completely ionized, acting as a strong acid:



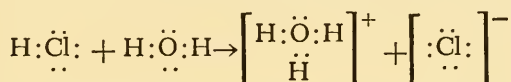
It is obvious that the behavior of a substance as an acid or base depends greatly upon the solvent medium. Many substances considered to be strong acids because of their behavior in water may well be weak acids in another solvent or may even be bases. For example, hydrochloric acid is only slightly ionized in benzene while nitric acid and water act as strong bases with sulphuric acid as solvent,



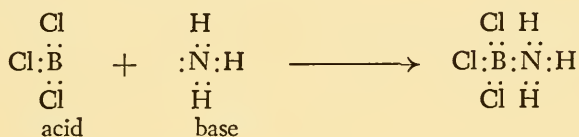
This broader view of the acid-base relationships has an important advantage in understanding the catalysis of many reactions. Many

substances other than hydrogen or hydroxyl ions are expected to act as acids or bases from this viewpoint and this realization has clarified the picture of a great number of complex reactions.

Often the even broader concept of acids and bases proposed by G. N. Lewis is called upon. According to this theory, substances which accept a share in an electron pair are acids, and the donor substances are bases. This picture not only includes as a special case the substances



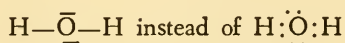
treated by the Brönsted-Lowry concept, but also extends to neutralizations involving no proton transfer at all:



These viewpoints of the nature of acids, bases, and solvents will be incorporated into the discussion of examples of processes where either specific acids or bases act as catalysts or where any acid or base would be effective to an extent dependent upon its strength. In the latter case, i.e., general acid-base catalysis, the symbols HA and A<sup>-</sup> will serve for acids and bases in general.

Furthermore, the course of some of the reactions will employ the concept of quantum-mechanical *resonance* to justify the existence of intermediate substances. Where this resonance stabilizes the intermediate, it will be shown in customary style by a double-headed arrow (↔) connecting the several canonical resonating structures which serve as our designation of the resonance hybrid.\*

In representing the electron pairs in certain of the diagrams, dashes are used. Thus



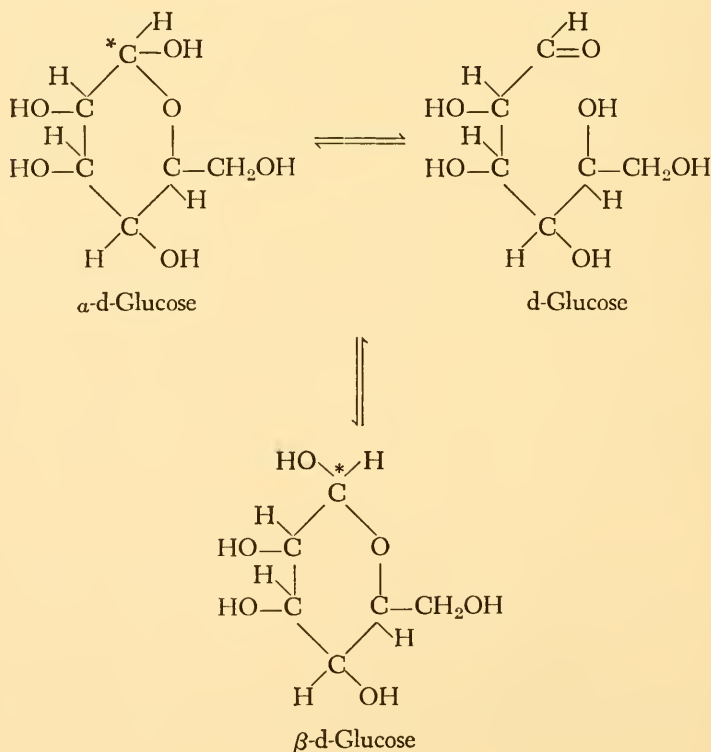
The hydrogen ion will often be written as a base proton, H<sup>+</sup>, although it is realized that the proton is solvated. This procedure is followed largely as a matter of convenience, but it should be pointed out that it is justified on other counts. First, the exact extent of

\*The reader is referred to such works as L. Pauling's *The Nature of the Chemical Bond* (1944) for discussion of the fundamental basis of the resonance phenomenon.

solvation is not known and it is really equally misleading to write a definite structure, such as  $\text{H}_3\text{O}^+$  for the hydrogen ion. Secondly, it is not general practice to show the solvation of other ions, although nearly all ions are solvated because of the ion-dipole or ion-induced dipole forces present in their solutions. For these reasons the solvation will be shown only when it serves the purpose of clarifying the interaction of the solvent with the acids and bases present.

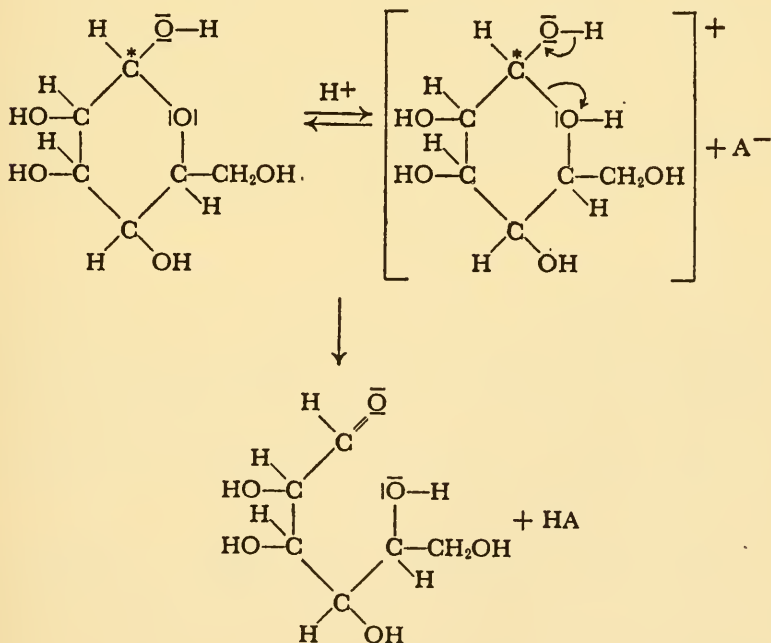
*General Acid and Base Catalysis.* The role of acid or base as a catalyst in reactions involving esterification, hydrolysis, condensations, and carbonyl addition reactions is to accentuate the difference in electron density between the two reacting centers, as indicated in the following examples.

**a. Mutarotation of Glucose by Acid and Base Catalysis.** It is known that when glucose is dissolved in water its specific rotation is  $+110^\circ$  which gradually sinks to  $+52.5^\circ$  on standing. This change, known as mutarotation, is the result of the transformation of  $\alpha$ -glucose



( $+110^\circ$ ) into  $\beta$ -glucose ( $+19^\circ$ ), and *vice versa*, until an equilibrium (at  $+52.5^\circ$ ) is established, *i.e.*  $\alpha$ -glucose  $\rightleftharpoons$   $\beta$ -glucose. In  $\alpha$ -glucose the hydroxyl groups at  $C_1$  and  $C_2$  are in *cis*-, and in  $\beta$ -glucose in *trans*-positions. The mechanism of the interconversion of d-glucose,  $\alpha$ -d-glucose, and  $\beta$ -d-glucose has been extensively studied.

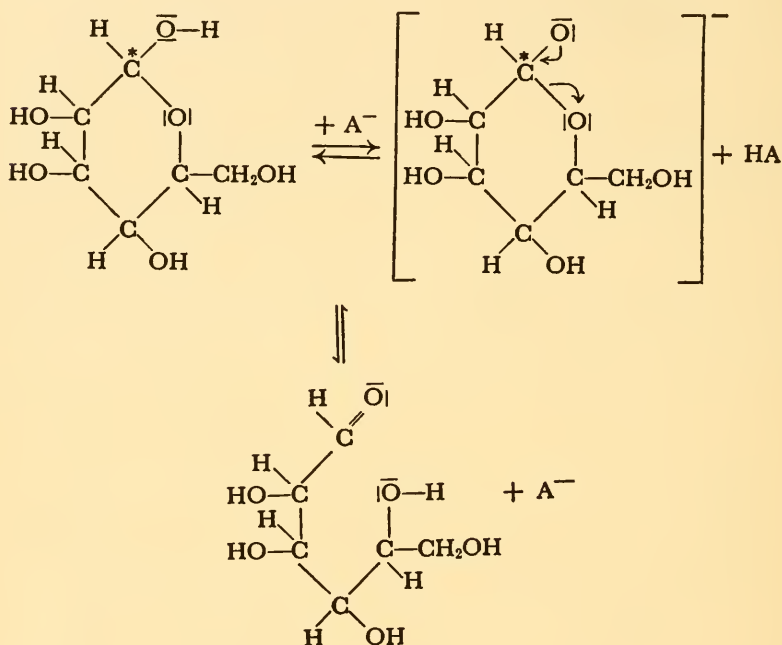
The mutarotation of glucose involves the reversible change of one stereoisomeric form of glucose into another which differs only in the spatial arrangement of the groups attached to the carbon marked with \*. This reaction requires the breaking and the reformation of one of its bonds. It has been shown that it is not the carbon to hydrogen link which has been severed, for if the mutarotation takes place in  $D_2O$  this hydrogen is not exchanged by deuterium. It has therefore been assumed that the ether oxygen to carbon link is involved in this conversion. According to the general *acid-base catalysis* the reaction proceeds in the following manner.



In the acid catalysis there is mobile and reversible addition of a  $\text{H}^+$  to the ether oxygen which is followed by a comparatively slow reaction with a base ( $\text{A}^-$ ) producing the symmetrical carbonyl form. While in

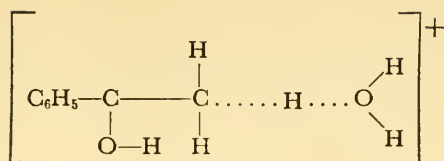


the base-catalyzed reaction the hydrogen of the hydroxyl group attached to the carbon (\*) linked to the ether-oxygen is first reversibly removed by the catalyst base. Then an acid reacts at a much slower rate with the anion formed to produce the symmetrical form.

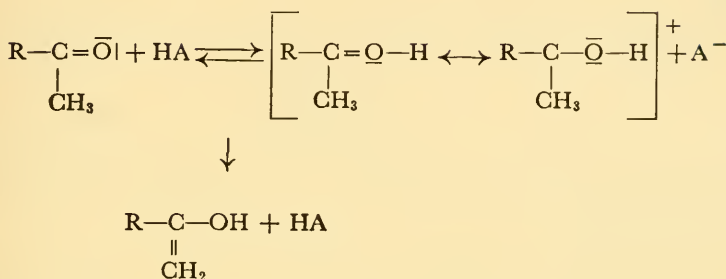


As can be seen, both an acid and a base are needed to catalyze the mutarotation. In this reaction there is an addition as well as the removal of a hydrogen ion. In either case, the conversion of the carbonyl form of glucose to the ring form may take place in either configuration of the carbonyl carbon, and this is responsible for the change to an equilibrium mixture of  $\alpha$ - and  $\beta$ -glucose, with a change of rotation to that of the mixture (Hammett, 1940, p. 337).

**b. Acid Catalyzed Enolization. Enolization in Concentrated Aqueous Acid.** As an example of enolization in concentrated aqueous acids again the primary reactions involve the formation of the conjugate acid of the ketone which in turn loses its  $\text{H}^+$  to a molecule of water as a base. Hammett (1940, p. 276), believes that in strong acid this molecule of water forms part of the complex in the transition state of the enolization of acetophenone.

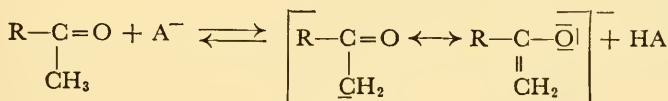


### c. Enolization in Dilute Acid.

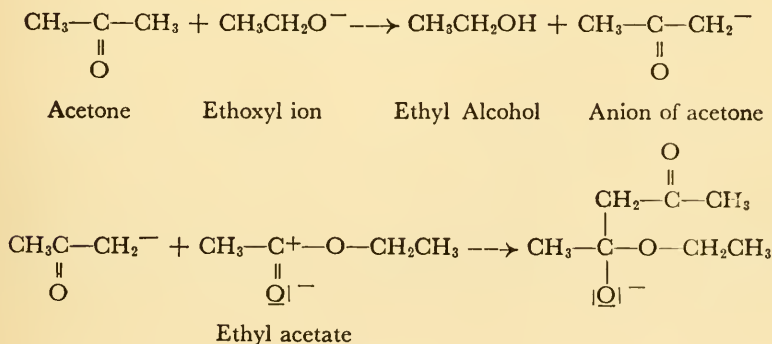


The enol may change to the ketone and since it has a symmetrical ion as an intermediate this must lead to the racemization of the ketone in case the  $\alpha$ -carbon is asymmetric.

In base accelerated reactions of such ketones, satisfactory explanation of the course of the reaction involves the reversible formation of a very reactive carbanion as follows:

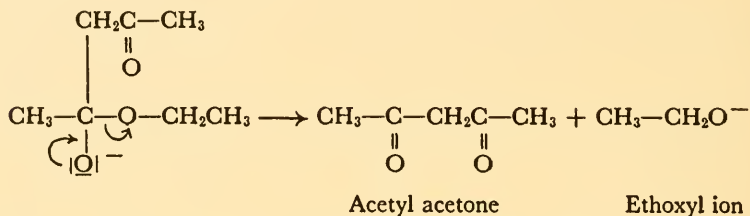


### d. Acid-Base Catalysis of Condensation Reactions. Base Catalysis of Condensation Reactions.



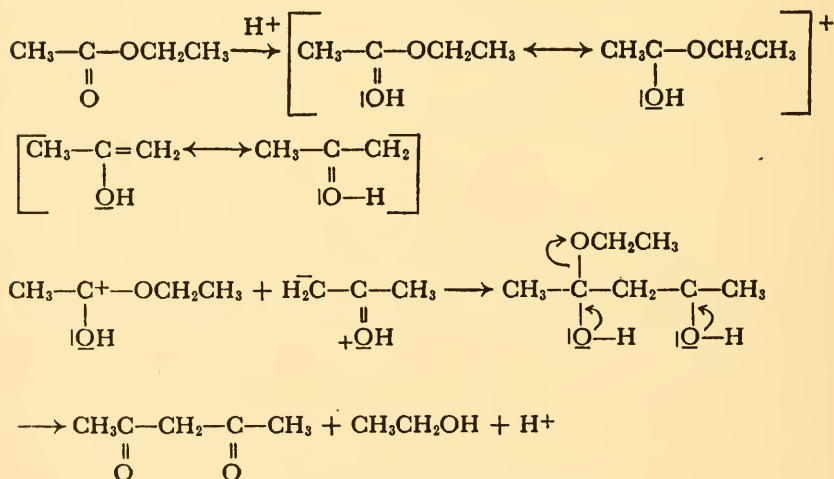


In the above reaction, ethoxyl ion functions as basic catalyst and removes a hydrogen from acetone making it electron rich (anion of acetone). This ion attacks the carbonyl carbon of the ester forming a new carbon to carbon bond.



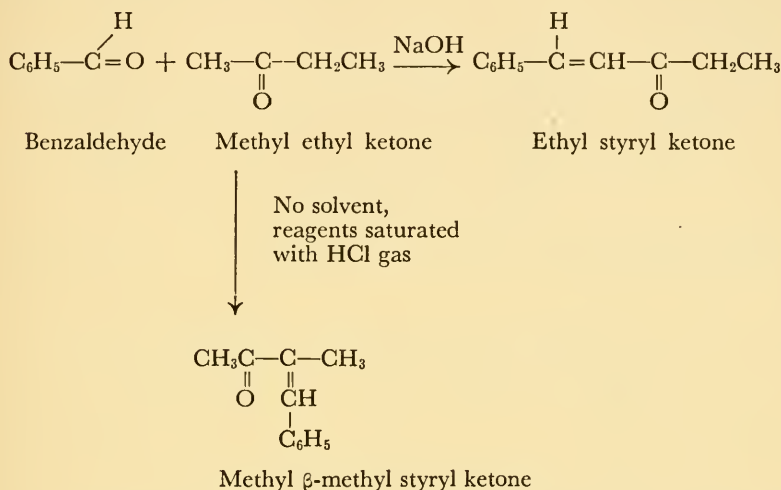
The pair of electrons on the oxygen of the carbonyl carbon will displace the ethoxyl group regenerating a new ethoxyl ion which can function as catalyst.

**e. Acid Catalysis of Condensation Reactions.** In the acid catalysis of the above condensation reactions, the same products are obtained, as shown in the following equations.



In the acid catalysis the  $\text{H}^+$  will attach itself to one of the lone pairs of the carbonyl oxygen of the ester thus making it positively charged, which in turn draws out one pair of the double bond electrons of the carbon and oxygen bond thus rendering the carbon electron deficient and vulnerable to attack by the electron-rich methylene group of the acetone molecule.

However, in the case of unsymmetrical reactants different reaction products are obtained by acid and base catalysis shown below (Hammett and Gettler, 1943).



**f. Hydrolysis of Esters.** Carboxylic ester hydrolysis reactions are slow reversible reactions catalyzed by both hydroxyl and hydrogen ions. The alkaline hydrolysis of an ester is a reaction between the ester molecules and hydroxyl ions, while in the acid catalyzed reaction a molecule of water may form a part of the transition state complex.

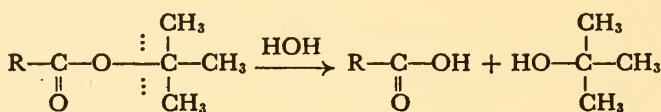
*Base Catalyzed Hydrolysis.* In this hydrolysis the carbon-oxygen bond that breaks is between the carbonyl carbon and oxygen and not the *alkyl carbon and oxygen*. This is based on the following observations (Hammett, 1940, p. 354).

(a) Using amyl acetate in water containing labeled oxygen ( $\text{O}^{18}$ ), Polanyi and Szabo (1934) showed that oxygen of the alcohol formed by the alkaline hydrolysis of the ester is the original ester oxygen and did not come from the water.

(b) Alkaline hydrolysis of an ester containing an optically active alkyl group does not yield an inverted carbinol as should be expected if the bond between the oxygen and the asymmetric carbon of the alkyl group had been ruptured.

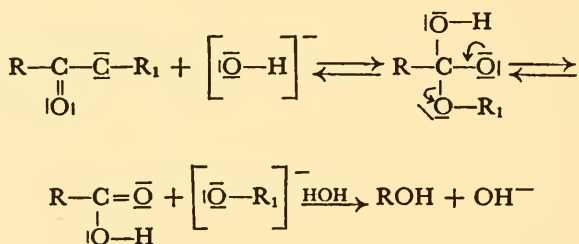
(c) No rearrangement takes place during the hydrolysis if the alcohol part of the ester consists of an  $\alpha$ -,  $\beta$ -unsaturated alcohol, thus eliminating the carbonium type reaction.

The hydrolysis of the esters derived from tertiary butyl alcohol forms an exception to the above mechanism in that the alkyl-oxygen linkage rather than the acyl-oxygen linkage is ruptured, as shown below.

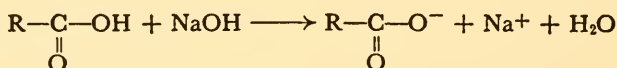


(Cohen and Schneider, 1941)

The hydrolysis can be represented as follows:

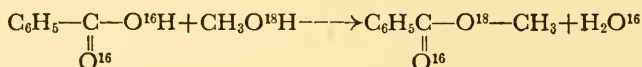


Alkaline hydrolysis of an ester is practically irreversible on account of the exhaustion of the anion due to the reaction:



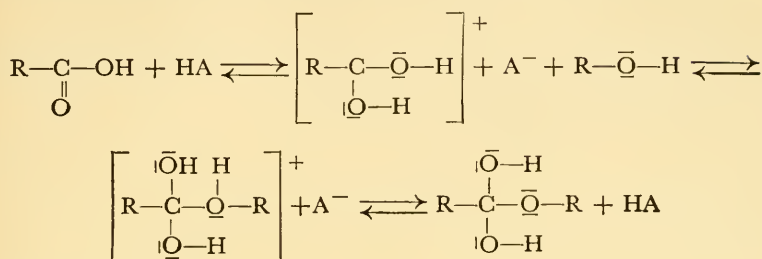
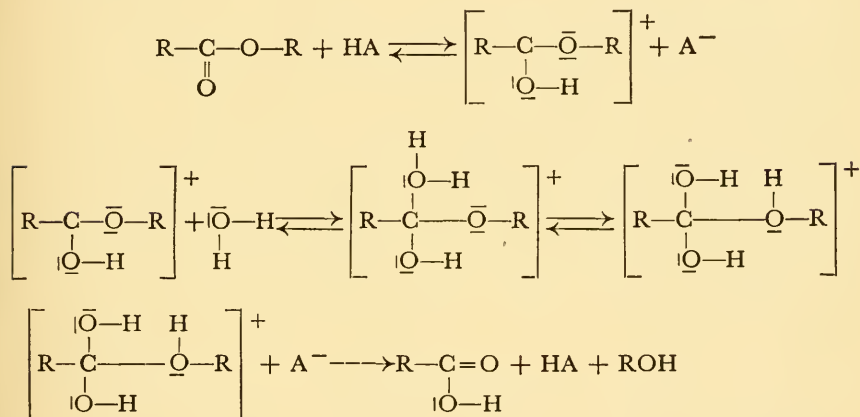
As an exception to this mechanism, in esters of sulphuric and sulphonic acids it is the alkyl oxygen linkage that is severed. In fact, these esters are used as alkylating agents.

**g. Acid Catalyzed Esterification and Hydrolysis.** Acid catalyzed esterification and hydrolysis are reversible and may go towards completion either to the right or left depending upon the excess of water or alcohol. In hydrolysis, the alkyl-oxygen link is not the one that is severed (Roberts and Urey, 1938, 1939), since it is shown by labeled oxygen studies that in esterification the ether oxygen of the ester comes from alcohol as shown below (Hammett, 1940, pp. 356-357).



*Esterification*

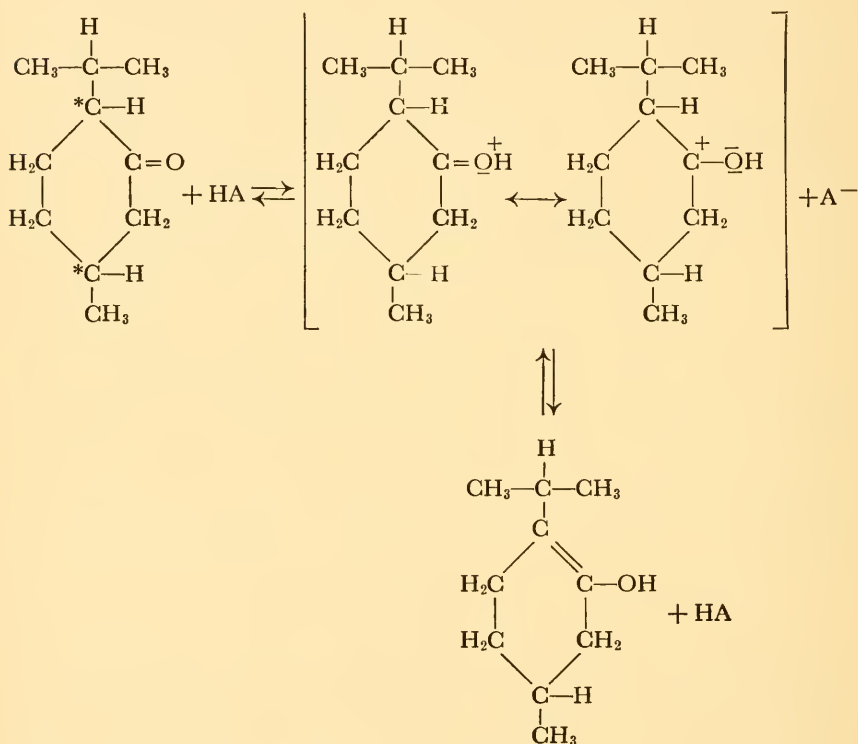
Detailed presentation of the esterification is given below:

*Hydrolysis*

## 2. Acid Catalysis in Non-Aqueous Solvents (Aprotic)

Acid catalysis in non-aqueous solvents such as chlorobenzene have been investigated. The rearrangement of *N*-bromacetanilide, the inversion of 1-menthone and the racemization of  $\text{C}_6\text{H}_5\text{COCH}-(\text{CH}_3)\text{C}_6\text{H}_5$  and  $\text{C}_6\text{H}_5\text{COCH}(\text{CH}_3)\text{CH}_2\text{CH}_3$  are some of the examples. The reactions are not kinetically of *integral order* but are fractional. With respect to the substrate the order is *less than one*. This can be attributed to hydrogen bonding type of complex formation between substrate and acid if the complex is chemically unreactive. Direct evidence has been found (Bell and Caldin, 1938) for the rapid and reversible formation of complexes of menthone with various acids resulting in the depression of optical rotation.

For example, in 0.036M trichloroacetic acid the  $[\alpha]_D$  of menthone was  $21.1^\circ$ , and in 0.762M acid the  $[\alpha]_D$  was  $11.3^\circ$ . This depression of optical activity was dependent on acid strength; for there was hardly any change in weak acids such as acetic acid, indicating that there was very little complex formation. The inversion of menthone may schematically be represented as follows:



The examples presented here suffice to give a general idea about quite a large class of chemical changes which occur generally according to the mechanisms underlying the examples cited.

From the standpoint of the subject matter the most fundamental aspects of these changes are: in the first place, when a chemical change takes place, a new substance is formed; secondly, the change is stoichiometrical, for every  $\alpha$ -d-glucose molecule which undergoes mutarotation a molecule of  $\beta$ -d-glucose is formed; thirdly, these changes are accelerated by catalytic agents, and none of the final iso-

meric forms, or two or more isomeric forms at a state of equilibrium contain the catalyst as part of the molecule.

Let us turn now to the production of antibody in the light of what we know about chemical changes. As discussed in the preceding pages, if the production of a new substance is brought about by the union of two reacting substances, then the reaction product or products should contain parts of the reactants according to stoichiometrical relationship. If on the other hand, such a reaction is accelerated by the presence of a substance which does not enter into the union irreversibly with the reaction products, the rôle of this substance is one of catalytic acceleration.

The absence of the catalyst or its parts in the final products of a catalytically accelerated reaction is one of the fundamental criteria of catalysis. If it can be demonstrated that the antibody formed in response to an antigenic substance does not contain the antigen molecule or its parts, the formation of the antibody can be assumed to have taken place by the antigen acting as catalyst. If, on the other hand, a chemical union has taken place between the antigen and the tissue substances forming the antibody complex, then this complex must be produced in accordance with stoichiometrical principles. The answer to this question must come from analytical and quantitative studies.

### 3. Characteristics Which Are Common to Inorganic Catalysts, Enzymes and Antigens

Before we present the facts concerning the quantitative relationship between antigen and antibody produced, let us discuss what a catalyst is. For later discussions it is also necessary at this point to specify the difference that exists between an inorganic catalyst on the one hand, and the enzymes or biocatalysts on the other. The inorganic catalysts, no doubt, exist in nature and are produced under natural conditions. However, modern chemistry has produced quite a large number of inorganic catalysts in the laboratory as the need may have demanded, or they are accidentally discovered. These are, therefore, artificial catalysts. All the enzymes or the biocatalysts without exception, on the contrary, are produced by living systems. An enzyme is a complex organic substance produced by a living cell and utilized by the cell



during the activity of its life-cycle. It would therefore seem quite reasonable to assume that the special physiology of each type of cell is controlled by the specificity of the cellular enzymes. It is to be noted, however, that the catalytic activity of an isolated enzyme is generally independent of the living processes of the cell which produce it, although the activity of such an enzyme as part of the cellular system may be different, or a great deal more active, than when it acts as a single isolated chemical entity.

*Crystalline Enzymes.* Pasteur and his followers regarded the catalytic activity of biological systems as an inseparable part of the phenomenon of life, and outside of experimental science. Liebig challenged Pasteur's point of view and advocated the existence of enzymes outside of the cell. Büchner in 1897 settled the argument finally by demonstrating that the fermentation of sugar could be caused by yeast extracts free from living cells; since then, not only many cell-free enzyme preparations have been obtained, but a score of enzymes has been obtained in crystalline form, which fact establishes, beyond doubt, the chemical individuality of enzymes. Crystalline *urease* (Sumner, 1926) was the first enzyme obtained in crystalline form. Later many crystalline enzymes and their precursors have been obtained and their purity studied by critical methods. They are: *pepsin* (Northrop, 1930); *amylase* (Caldwell, Booher and Sherman, 1931); *yellow enzyme* (Warburg and Christian, 1932; Theorell, 1934); *Chymotrypsin* (Kunitz and Northrop, 1935); *carboxypeptidase* (Anson, 1935); *ficin* (Walti, 1937); *papain* (Balls, Line-weaver and Thompson, 1937); *lysozyme* (Abraham and Robinson, 1937); *catalase* (Sumner and Dounce, 1937); *alcohol dehydrogenase* (Negelein and Wulff, 1937); *tyrosinase* (Dalton and Nelson, 1938); *lecithinase* (Slotta and Fraenkel-Conrat, 1938); *d-ribonuclease* (Kunitz, 1939; muscle *d-glyceraldehyde-3-phosphate dehydrogenase* (Warburg and Christian, 1939); yeast *d-glyceraldehyde dehydrogenase* (Krebs and Najjar, 1948); muscle *lactic dehydrogenase* or *pyruvic acid reductase* (Straub, 1940); *peroxidase* (Theorell, 1940); *fumarase* (Laki and Laki, 1941); *Rennin* (Hankinson, 1942); *phosphorylase* (Green, Cori and Cori, 1942); *phosphate transporting enzyme*, 1,3-diphosphoglyceric acid + adenosine diphosphate  $\rightleftharpoons$  3-phosphoglyceric acid + adenosine triphosphate (Schelling, 1942); *myosin* (Szent-Györgyi, 1943); serum mucoprotein with high *cholinesterase* activity (Bader,



Schultz and Stacey, 1944); yeast *hexokinase* (Kunitz and McDonald, 1946); *lipoxidase* (Theorell, Holman and Akesson, 1947); bacterial  *$\alpha$ -amylase* (Meyer, Fuld and Bernfeld, 1947); pancreatic *desoxyribonuclease* (Kunitz, 1948).

The fundamental characteristic of all catalyzed processes is that they are reactions which, in the thermodynamic sense,\* are classed as spontaneously occurring processes. That is, they are reactions which occur with diminution of free energy. Any added substance which can accelerate such a reaction is known as a catalyst. A catalyst accelerates slowly progressing reactions and enables them to attain the equilibrium condition in a very much shorter time. The equilibrium point is not changed but the time necessary to attain this point is shortened.

To illustrate this point we may use the following example. We know that iron can combine with oxygen to form various iron oxides.

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\*The thermodynamic criterion for a reaction to occur spontaneously is that it do so with a loss in *free energy* of the system, change in *free energy* being the difference of the *free energies* of a system in its initial and final states. Hence, any system, left to itself, will change in such a way as to approach a point of equilibrium where the change in *free energy* will equal zero. Thus, for example, in a system consisting of two bodies at different energy levels there will be transfer of energy from one to the other at different rates. The body at the higher energy level, transferring energy at a greater rate, tends to raise the energy level of the second body until a condition is reached where both bodies are at the same energy level. No energy is thereby lost, but the capacity for spontaneous change has vanished, and the system is said to have less *free energy*. *Free energy* is a function which indicates the direction in which chemical or other processes take place, and is of great theoretical value in fixing the conditions of *equilibrium*. The fundamental conception is that of a *reversible cycle* of operations. The condition of *reversibility* is that the state of the system at any time does not differ sensibly from equilibrium, for then the slightest variation in the conditions will determine the occurrence of the process in the one direction or the other.

Kinetic theory postulates that chemical reactions take place only when molecules collide. However from chemical kinetics it has been demonstrated that only those collisions between reactants are effective in which the joint energy contributed by the molecules is equal to or greater than a certain minimum energy value termed *energy of activation*.

A catalyst does not affect the point of equilibrium but accelerates the rate at which the equilibrium state is attained. Thermodynamically this may be expressed by saying that the change of free energy involved in a chemical reaction is the same whether a catalyst is present or not. Kinetically, this means that the catalyst accelerates the rate of the reverse reaction to the same extent as that of the forward reaction, so that the equilibrium constant, equal to the ratio of these rates, is the same for the catalyzed and uncatalyzed reactions. The function of the catalyst is to bring about the desired reaction with a smaller energy of activation. A lower energy of activation gives a more rapid reaction because more molecules have the necessary amount of energy to react. The high energy requirement is avoided by some by-pass. Usually the by-passing consists in forming an unstable *activated complex* with less energy consumption and then decomposing this intermediate compound in such a way as to regenerate the catalyst. In this way the catalyst is used over and over again.

But under completely dry conditions a noticeable combination will not be observed. They will, however, combine on ignition forming oxide of iron. The applied heat accelerates the combination till sufficient heat of reaction is produced to make it self-sustaining. On the other hand, when we bring oxygen and iron together in the presence of moisture at ordinary temperature the reaction starts automatically, the moisture acting as catalyst. The rôle of moisture here was to reduce the *energy of activation* necessary for the attainment of the equilibrium of the reaction between oxygen and iron.

As previously mentioned, there is no stable union between a catalyst and the reaction products or reactants although it may form intermediate, reversible and labile complexes with these substances; but the lives of such complexes are very short; the combination is fugitive. It is stated that the mean life of an active  $\text{H}_2\text{O}_2$ -catalase complex (catalase contains trivalent iron) is  $1.2 \times 10^{-7}$  second. This mean life is unique in being derived from purely chemical data, and does not involve any hypothesis as to collision. Likewise, the mean life of the excited oxyhaemoglobin complex (contains bivalent iron) is  $4.0 \times 10^{-6}$  second (Haldane, 1931).

The fact that a minimum amount of a catalyst is capable of transforming a large quantity of substrate is understandable for the above mentioned reason that after the breakdown of the catalyst-substrate complex the catalyst can complete another cycle of acceleration, and continuously thereon. It is true that the unchangeability of an ideal catalyst during a reaction is one of its essential characteristics and responsible for its continued activity, but it does not need to be so, for when a catalyst takes part in a reaction and establishes the equilibrium it is possible that a certain amount of catalyst is tied by the reaction products as specific inhibitors, or used up by the system so that its redelivery from the theoretical equation: (A and B reactants,



C catalyst, AB reaction products, AC catalyst-reactant complex) is not complete. Also the catalyst might chemically combine by a side-reaction and thus be removed from the field of action. The catalyst might be destroyed by one of the reaction products, such as the destruction of the oxidative enzyme system of xanthine oxidase or pneumococcus by the hydrogen peroxide formed as one of the reaction products. Addi-

tion of a little catalase at the outset to the reaction system decomposes hydrogen peroxide as soon as it forms, and thus the enzyme can continue functioning for many hours without weakening.

a. **Disproportionality between the Amount of Inorganic Catalysts and the Amounts of Substrates Catalyzed.** Following the above discussion regarding the general and qualitative aspect of these properties of catalysts let us now present a few quantitative data. The combination of hydrogen and oxygen at ordinary temperature could be brought about by 2.5 ml. of a colloidal solution of platinum containing as little as 0.17 milligram of platinum, and at the outset the rate of combination was 1.8 ml. of gas per minute. After a period of time during which 10 liters of gas had undergone combination it was found that the activity of the colloidal solution was still unimpaired (Rideal and Taylor, 1926). The spontaneous slow decomposition of  $\text{H}_2\text{O}_2$  can be accelerated by colloidal platinum in a dilution of 1 Mol (194 g. of platinum) in 70,000,000 liters. The presence of 0.000,000,000,0001N.  $\text{CuSO}_4$  solution is sufficient to produce a perceptible acceleration of the rate of oxidation of an aqueous solution of sodium sulphite (Titoff, 1903). The oxidation of aniline hydrochloride, in the preparation of aniline black, is carried out in the cold by a solution of potassium or sodium chlorate with the aid of metal catalysts, the most active of which is *vanadium pentoxide*,  $\text{V}_2\text{O}_5$  of which one part is sufficient for 270,000 parts of aniline and the corresponding amount of chlorate (Sabatier, 1922).

b. **Disproportionality between the Amounts of Enzymes and the Amounts of Substrates Catalyzed.** Calculations (Haldane, 1931) show that one molecule of liver catalase (or one trivalent atom of catalase iron) decomposes ( $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$ )  $5.42 \times 10^4$  molecules of  $\text{H}_2\text{O}_2$  per second, at  $0^\circ\text{C}$  and  $10^{-2}$  M substrate concentration. Under the same conditions one molecule of plant catalase decomposes  $1.7 \times 10^5$  molecules of  $\text{H}_2\text{O}_2$  per second. One atom of peroxidase iron decomposes  $10^4$  molecules of  $\text{H}_2\text{O}_2$  in one second (Kuhn, Hand and Florkin, 1931). The iron of the cytochrome oxidase (Warburg) manifests an activity of about  $10^5$  oxygen molecules per second (Warburg and Kubowitz, 1929); one molecule of saccharase hydrolyzes  $7.0 \times 10^3$  molecules of sucrose per second (Moelwyn-Hughes, 1933); one part of pure solid carbonic anhydrase ( $\text{H}_2\text{CO}_3 \rightarrow \text{CO}_2 + \text{H}_2\text{O}$ ) in 7,000,000 parts of solution is sufficient to double the rate of  $\text{CO}_2$  evolution and

during the first 15 seconds 1 g. of enzyme is responsible for the production of 825 g. of  $\text{CO}_2$ , at a rate of 1.24 moles  $\text{CO}_2$  per sec. per g. of enzyme (Roughton, 1934); urease crystals possess an activity of a little more than 100,000 units per gram, or 100,000 mg. of ammonia nitrogen is produced in five minutes when acting on urea at  $20^\circ\text{C}$ . at pH 7.0 (Sumner, 1932); solutions made of crystalline trypsin or pepsin containing less than  $1/1,000,000$  (0.000,005 M) of a gram of protein nitrogen per ml. have an accurately measurable effect on the digestion of casein, while solutions of pepsin containing less than  $1/10,000,000$  of a gram of protein nitrogen have a very powerful effect on the clotting of milk (Northrop, 1932). One g. of crystalline pepsin dissolves 50,000 g. of boiled egg white in two hours, clots 100,000 liters of milk and liquefies 2000 liters of gelatin during the same period of time. One g. of purified rennin (Tauber and Kleiner) clots 4,500,000 g. of milk in ten minutes.

**c. Disproportionality between the Amount of the Antigen Used and the Amount of Antibody Produced.** The measurement of the amount of antibody produced in response to a given amount of antigenic substance encounters considerable technical difficulties, some of which are almost insurmountable. These difficulties are: (1) although the circulating antibodies could be approximately estimated by precipitin and agglutination reactions, we have as yet no way of determining the amount of the antibody fixed in the tissues; (2) we cannot determine the actual amount of antigen responsible for the antibody produced; it is not unlikely that only a fraction of the injected substance is instrumental in the production of antibody; (3) when whole organisms are used we have no idea of the number of antigenic molecules in an injected quantity of bacteria. Despite these difficulties the prevalent opinion among immunologists and bacteriologists is that there is a striking disproportion between the quantity of antigen and the total amount of the resulting antibodies.

As early as 1893 it was demonstrated (Roux and Vaillard, 1893) that continuous bleeding of horses actively immunized against tetanus toxin did not diminish the antitoxin content of the regenerated blood. In similar studies (Salomonsen and Madsen, 1898) on diphtheriae toxin used to immunize horses, the above observation was confirmed. Much more interesting was the observation that when the antibody diminished, or nearly completely disappeared, the administration of



non-specific substances, such as pilocarpin stimulated the restoration of the antibody formation. One unit of tetanus toxin produced (Knorr, 1898) 100,000 neutralizing antitoxin units in horses. A man, surviving a typhoid infection, would still contain in his blood agglutinins for months and years, in spite of the fact that a certain fraction of the antibody would be eliminated (Friedberger, 1902) through urine, or other routes. It was shown (Friedberger and Dorner, 1905) that bacteriolysins are produced in rabbits by 1/1000 of a loopful of cholera vibrios killed at 60°C., and antibodies in rabbits by injecting a total of 0.5–1.0 mgm. (300,000–900,000) goat red blood cells.

In the light of some of the above facts concerning the disproportionality between the very small amount of antigen used and the many fold quantity of antibody formed, the Büchnerian hypothesis, which considers the formation of antibody as a consequence of chemical union between antigen and the antibody, loses its significance (Müller, 1917). Newer and more accurately obtained quantitative data will be presented below concerning this question.

Seibert (1925) found waters spontaneously became contaminated after standing at least four days under non-sterile conditions. Bacteriological tests revealed the presence of chromogenic and non-chromogenic, motile and non-motile bacteria. Four liters of water on concentration was found to contain 0.036 mg. of nitrogen (after subtracting the blank). She calculated that 1 ml. of fever-producing water would have contained 0.000,000,005 g. of protein.

Ninhydrin tests on one liter concentrates of highly potent water were negative, as were also the tests on several bacterial filtrates.

*Immunological Studies.* A rabbit was injected with 1/50 ml. of water. After an elapse of 12–14 days it was injected with 10 ml. of the same water; within one hour and ten minutes it developed typical shock symptoms, e.g. scratched its nose, fell to one side paralyzed, collapsed, expelled bloody urine, gasped for air and died with violent convulsions. Autopsy revealed an enlarged right heart and congested liver. Several other experiments gave her similar results. Immunization of the rabbits with traces of solid material present in 20 ml. of non-sterile distilled water with a protein content of 0.000,062 to 0.000,12 g. produced an agglutinating serum which was active in dilutions of 1:8000 against bacteria found in such distilled water.

Branham and Humphreys (1927) found that the serum of animals

immunized with sterile filtrates obtained from *B. enteritidis* cultures incubated in a synthetic medium for six to 14 days contained agglutinins, precipitins and complement fixing antibodies. They could not demonstrate the presence of protein in these filtrates by the biuret, ninhydrin and Molisch tests; Millon's test for tyrosine and Ehrlich's and vanillin tests for tryptophane were negative.

Ten liters of toxic filtrate were concentrated *in vacuo* at 40°C. to a syrupy fluid of 200 ml. The protein tests still were negative, although the residue was still toxic for rabbits. After dialysis of the residue against distilled water the dialysate was concentrated *in vacuo* at 30° to 40°C. to a volume of about 5 ml. This concentrate showed by ninhydrin, vanillin, and diazo tests (the last for histidine) very faint, but definitely positive reactions. By comparing the sensitiveness of these tests to the positive reactions shown by the concentrate they calculated the protein content of the original solution at least as 0.000,000,1 g. per ml. of filtrate. Rabbits on immunization with 30 to 40 ml. filtrates containing a total of 0.000,003 to 0.000,004 g. of protein elicited definite antibody formation. The authors in the light of their work suggest that such infinitesimal amounts should be borne in mind in interpreting results obtained with apparently protein-free materials.

Topley (1930) evaluated as nearly as possible the relation between the amount of antigen injected into rabbits and the resulting agglutinin titre. The antigenic material consisted of a saline suspension of *Bact. paratyphosum* B. in the type phase, and the antibody studied was the corresponding H or flagellar agglutinin. Bacterial suspensions containing 0.25 per cent formalin were killed by heating at 55°C. for one hour. A series of rabbits were injected intravenously with  $10^8$ ,  $10^5$  and  $10^4$  bacilli per kilo body weight (k.b.w.); specimens of sera were collected at intervals of three to seven days during the first few weeks and at longer periods thereafter. The whole period of observations varied from 50 to 300 days or more. In estimating the response to inoculation two values were noted: (a) the highest titre attained, and (b) the mean titre during the first 50 days after inoculation. The calculations were made from the graph drawn from the actual observations.

The tabulated results show that with a single injection into each of three rabbits of a dose of  $10^8$  bacilli per k.b.w. they attained an immune serum with a highest agglutinating titre of 2550 to 4480 and

a mean titre during the first 50 days from 880–1630 for the three rabbits; and with a single dose of  $10^5$  bacilli per k.b.w. three rabbits showed a highest agglutinating titre of 130 to 510 and a mean titre of 37 to 280 during the first 50 days for a second series of rabbits. Thus Topley finds that a dose of  $10^5$  bacilli per k.b.w. is in the neighborhood of the threshold dose for a detectable response.

To determine the actual amount of antigen contained in a dose of this order a dense bacterial suspension was dried and brought to constant weight over calcium chloride; and the dry weight of  $10^9$  bacilli was found to weigh 0.55 mg. A dose of  $10^5$  bacilli thus corresponded to about 0.000,005 mg. of solid material. When allowance had been made for the possible presence of non-bacterial material brought in with bacteria by washing out the agar in preparing the suspensions, and for the flagellar material which could not have contained very much antigen, it would seem probable that the dose of active substance administered with  $10^5$  bacilli was of the order of 0.000,000,5–0.000,005 mg. After such a consideration Topley used a conservative figure of 50 ml. of serum contained in the blood of a rabbit of 2000 g. and evaluated its agglutinating titre as 1:500 or more. He thus presented a quantitative study to emphasize the well known and striking disproportionality between the amount of antigen injected and the amount of antibody produced.

Hooker and Boyd (1931) using the data obtained by Topley carried out calculations to determine the amount of antibody produced by a single antigen molecule. They assumed that the above mentioned weight of material—as evaluated by Topley—present in  $10^5$  bacteria as the minimal effective dose of active antigenic substance is of the order of  $5 \times 10^{-10}$  gram per k.b.w. of rabbit having an agglutinating titre of serum 1:500. Considering that the fundamental specific antigenic unit has a molecular weight of 20,000—a very conservative figure—they arrived at the following conclusions:

One gram of material will contain, after Avogadro,  $5 \times 10^{-5}$  ( $6.06 \times 10^{23}$ ) =  $3 \times 10^{19}$  molecules, and the minimal effective dose of antigen ( $5 \times 10^{-10}$  g.) per k.b.w., contains therefore approximately  $1.5 \times 10^{10}$  molecules.

One ml. of serum from rabbit 11 responsive to  $8.3 \times 10^{10}$  molecules in Topley's experiments contained 5000 or more agglutinin units. A "unit" was estimated to affect the agglutination of  $2 \times 10^8$  bacteria



or a much larger number of flagella. Fifty ml. of serum (estimated conservatively by Topley) obtainable from the 2760 g. rabbit would yield  $2.5 \times 10^5$  units of agglutinin, resulting from the stimulus of  $8.3 \times 10^{10}$  antigen molecules, and capable of flocking  $5 \times 10^{13}$  bacteria. In other words, one molecule of antigenic substance gives rise to an amount of circulating agglutinin capable of flocking 600 bacteria. As they state, this evaluation does not take into account the large reservoir of antibody in tissue fluids and cells which by repeated daily bleeding would contribute in no small measure to a magnification of this figure.

Studies by Heidelberger and Kendall (1930), and Heidelberger, Kendall and Soo Hoo (1933) likewise demonstrated the striking disproportionality between an artificial antigen and the amount of antibody produced. The antigenic substance was colored R-salt-azobenzidine-azo-crystalline egg albumin. The injections of the antigen were carried out either in solution, or adsorbed on collodion or alum particles. Fifty ml. of a suspension of antigen adsorbed on collodion, the amount used for 16 injections, contained 0.55 mg. of dye and 140 mg. of collodion. In all but one series the injections were given intravenously, four daily injections each week for four weeks; many animals were given an additional course of two, three or four weeks.

Quantitative data showed that as much as 0.73 to 0.94 mg. of circulating antibody per ml. of serum may be formed in response to injections of antigen totaling 0.35 to 0.55 mg., or a total response for the rabbit of over 100 mg. of circulating antibody for every milligram of antigen injected. Since the ratio of antigen to antibody in the precipitate has been found to average 1:7 at the equilibrium point, the authors stated that at least 12 times as much antibody as necessary to combine with the amount of antigen used is formed. This evaluation, of course, does not take into account the antibodies present in the tissues as well.

Morgan (1937) extracted an antigenic material from *B. dysenteriae* (Shiga) by diethyleneglycol. This material contained a polysaccharide, gave positive biuret and negative ninhydrin reactions. On immunizing a horse with a total of 16.2 mg. of material the production of 1.64 mg. of antibody protein per ml. of blood serum was attained. Morgan assumes a blood serum volume of 25 liters obtainable from the immunized horse, or a total of 41 g. of antibody protein in response to 0.0162 g. of antigenic material, a ratio of (41/0.0162)

2500:1. His findings show also that the ratio of mg. of antibody produced per mg. of antigen injected, for rabbits that have been immunized was considerably smaller than the one obtained for the horse. In the five sera examined they ranged in the order of 550:1, 550:1, 200:1, 400:1, 1000:1, respectively.

Pappenheimer (1940) immunized a horse against egg albumin. During the entire course of injections the horse received a total of 1.9 g. of egg albumin. Assuming that the animal contained 25 liters of serum at the time of the final bleeding the total circulating anti-egg albumin antibody was estimated to be in the neighborhood of 50 g.

A horse was immunized with a total of 60 mg. (20,000 Lf. units) of diphtheria toxoid by Dr. W. B. Rawlings of the National Drug Company over a period of one month. At the end of this period the horse contained 2150 units of antitoxin per ml. of serum, equivalent to 25 mg. of antitoxin per ml. which was estimated (Pappenheimer, 1940) to correspond to more than 600 g. of total circulating antitoxin or more than 10,000 times the weight of antigen injected.

**d. Absence of Inorganic Catalysts, Enzymes and Antigens in the Catalyzed Reaction Products.** In the preceding pages several quantitative studies were cited demonstrating that the amount of a catalyst or an enzyme in ratio to the amount of the products of reaction they accelerate is incomparably small. Likewise, experimental data were cited demonstrating that the ratio of the amount of antibody produced to the amount of antigen used is strikingly disproportionate. These quantitative relationships can be interpreted to signify that the antigen could not have entered into stoichiometrical chemical union with  $\gamma$ -globulin to form the final antibody complex. Müller (1917), Heidelberger and Kendall (1930), Topley (1930), Hooker and Boyd (1931) have expressed this view in opposition to the Büchnerian hypothesis of antibody formation. Hooker and Boyd after having calculated that one molecule of active antigenic substance gives rise to an amount of agglutinin capable of flocking 600 bacteria, and that the surface relationship between one globulin molecule and 600 bacteria is 1:25,000,000, stated that "A theory of antibody formation involving catalysis would seem more promising."

If our assumption that antibody produced by catalytic acceleration of antigen is true, then we must be able to demonstrate chemically that antigen is actually absent in the antibody molecule. For when

colloidal platinum catalyzes the combination of oxygen and hydrogen with the formation of water, copper sulphate catalyzes the rate of oxidation of an aqueous solution of sodium sulphite, and urease the transformation of urea into ammonium carbonate, etc. no traces of catalysts are found in the molecules of the reaction products.

Doerr and Friedli (1925) were perhaps the first to undertake the task of showing the absence of antigenic substances in the antibody molecule by chemical analysis of the latter. By immunizing rabbits with atoxyl-containing azo-proteins, highly active anti-atoxyl specific immune sera were obtained. Analysis of these specific sera with a highly sensitive chemical method failed to show the presence of arsenic. On the basis of their findings they concluded that antibody cannot be a metabolite originating from the substance of antigen.

Following the above mentioned work Berger and Erlenmeyer (1931) diazotized the sodium salt of p-aminophenylarsenic acid (atoxyl) and coupled it with normal horse serum. The purified antigen, which contained 0.000,449 g. arsenic per ml. of solution, was used for the immunization of rabbits, administering four intravenous injections of 2.0, 4.0, or 6.0 ml. of antigen solution. The animals were bled 13 days after the last injection. The immune sera thus obtained reacted with the antigen solution of 1:3200–6400 dilution. Using 30 ml. of immune serum for the chemical detection of arsenic they did not find any trace of arsenic in two rabbit sera. The serum of a third rabbit showed a faint trace which was definitely weaker than a positive control containing  $10^{-8}$  g. (0.01 $\gamma$ ) arsenic. A rabbit weighing two kilos was calculated to contain 90 ml. of serum so that the arsenic content of the total serum in none of the three rabbits could have been 0.000,03 mg. (0.03 $\gamma$ ).

For the detection of the arsenic in serum they used a micro method based on the Marsh test whereby an amount of arsenic as little as 0.01 $\gamma$  ( $10^{-8}$  g.) could be demonstrated. In order to show whether arsenic was present in the serum they analyzed the sera of rabbits immediately—3½ to 20 minutes—after injecting them with atoxyl. Their positive findings thus showed that arsenic, if present, in serum can be determined under the experimental conditions. The evidence, therefore, appears to show conclusively that arsenic in antigen is not incorporated in the resulting antibody.

Hooker and Boyd (1932) immunized rabbits with casein-diazo-ar-

sanilic acid which had a calculated molecular weight of 114,000 and contained 78 diazo-arsanilic acid groups combined with one casein molecule. A calculation of the serological results showed that the ratio by weight of antigen to antibody was 1:18, or adopting a liberal figure, was at least 1:10.

Analyzing their quantitative data, they stated that if the most probable calculation is made, on the basis of the Büchnerian hypothesis, 15 ml. of immune serum should contain 15.3 $\gamma$  of arsenic. In actual experiments this volume of serum showed no trace of arsenic, though trial runs showed that the method would detect 1.0 $\gamma$  of arsenic.

In a study previously cited, Heidelberger, Kendall, and Soo Hoo (1933) immunized rabbits against a red azo dye, R-salt-azo-benzidine-azo-crystalline egg albumin. If the red colored whole antigen or its red prosthetic group had been incorporated into the antibody according to the Büchnerian hypothesis, the immune sera, or the isolated antibody should be colored. The immune sera from the rabbits showed no trace of color.

Wollman and Bardach (1935) showed the falseness of the Büchnerian hypothesis by a highly sensitive anaphylactic test. The fact that by means of an anaphylactic test the presence of a protein in a mixture of several proteins can be detected was used to determine whether or not a protein antigen, assumed by the theory to have been incorporated in the homologous antibody molecule would cause anaphylactic shock in guinea pigs sensitized against the same antigen. The guinea pigs sensitized against egg albumin and horse serum and injected with homologous protein suffered shock and death, but the sera of rabbits immunized against these antigens produced no symptoms of anaphylaxis in another group of guinea pigs sensitized respectively against egg albumin and horse serum.

Haurowitz, *et al.* (1942) found that homologous antibodies produced in rabbits to iodo-proteins, bromo-protein, caseinogen (phosphoprotein) and arsanil-azoprotein did not contain any of the determinant group of the antigen or a serologically related group.

In the absence of any other plausible explanation which could be offered at present, the most logical possibility therefore is that antibody production is directed by antigen acting as a catalyst at the site of the antibody formation. This point of view appears to meet the criteria of the well known catalytic processes employed in all branches



of chemistry. Before we proceed to the elaboration and discussion of other aspects of this problem, we must also discuss the question of whether or not enzymes are antigenic. For the production of a specific antibody in response to an antigen acting as a directive specific catalyst and the antigenicity of an enzyme of which the apparent function is to catalyze biological processes specifically, are intimately related.

**e. Enzymes as Antigens.** It is a generally accepted fact that the antigenic property of a substance, or more specifically, the production of antibody against an antigen is usually dependent on the protein molecule. It is also an accepted fact that the enzymic activity of a substance of biological origin is dependent on the presence of protein in the enzyme molecule. Even in those enzymes the specificity of which is associated with a prosthetic group, this group must necessarily be conjugated with a specific protein molecule or a "Kolloidal-träger" of protein nature. In its absence the prosthetic group is enzymically inactive. Likewise, when an antigen is conjugated with a non-protein group, such as atoxyl, the antigenicity is conditioned by the protein molecule, even though the prosthetic group may be the factor determinant of serological specificity.

From the standpoint of chemical composition, both antigen and the enzyme proteins are of the same nature. Generally speaking, their amino-acid content, physical properties, molecular size and their behavior to various chemical treatments are of the same nature, or order of magnitude. There is no theoretical basis, therefore, to exclude the enzyme proteins from the family of antigenic proteins.

The antigenicity of an enzyme protein can be determined by the well known serological methods: (a) by antigen-antibody reaction *in vitro* causing the formation of a precipitate, (b) by anaphylactic tests, and (c) by inhibition of the enzymic activity as a consequence of the combination between the enzyme and its specific antibody. Undoubtedly, other methods supplementary to one or the other method can also be employed. Of the methods the first and second will be discussed here briefly, and the third will be reserved for a later discussion when we come to the correlative treatment of the biological significance of antibodies against antigens (enzymes).

In characterizing the antigenicity of an enzyme only by the precipitation method, we must be aware of the fact that every enzyme preparation cannot be accepted as free of other protein impurities.

If a preparation contains an enzyme protein and an inactive protein impurity the immune serum produced against such a mixture will react in the precipitation tests with one or both of them; it will hence be difficult to state whether the precipitinogen was the enzyme or the contaminating protein. We will therefore include only immunological studies carried out using the more highly purified crystalline preparations.

*Antibody against Crystalline Urease.* Antiurease was the first antibody produced against a crystalline enzyme, and this is probably the first indubitable proof of the existence of an immune anti-enzyme. Sumner and Kirk (1931; Kirk and Sumner, 1931, 1932, 1934; Sumner, 1937; Howell, 1932) obtained antiurease which behaved like antitoxins prepared against bacterial toxins in precipitation and neutralization experiments. Antiurease serum inhibited the catalysis of urea into ammonium carbonate by urease, and protected animals against the toxic and fatal effects of ammonia resulting from the dissociation of ammonium carbonate.

Crystalline urease prepared from jack bean meal of high urease content had an activity of about 135 units per milligram. The crystals under the microscope were seen to be practically uncontaminated by any other material.

When 1 ml. of crystalline urease solution containing 100 units was injected into the ear vein of rabbits they experienced convulsions in a few minutes and death within an hour. When two rabbits were injected intraperitoneally or subcutaneously with 0.5 ml. (50 units) of solution death followed within 48 hours. To prevent the death of rabbits 2.5 to 5 units of urease were injected at the start. The injections were given every eight days for 30 days and then every two or three days for 30 days. The last injections contained 600 units of urease. The serum of rabbits showed the presence of precipitin, though of low titer. However, such a serum was capable of neutralizing the urease, and the urease-antiurease combination was used for immunization against urease. Animals therefore were usually started with a suspension of urease-antiurease in large doses (containing 100 to 1000 urease units, per animal). This indicated that a very effective protective antibody had been produced during the first immunization experiments. During the course of this study it was found that the recrystallized urease of highest purity gave better results than the once-crystallized urease.



The immune sera of the rabbits contained from 30 to 40 antiurease units per ml. and the amount of antiurease in the blood serum of an immune hen varied from five to 24 neutralizing units per ml. The rabbit immune serum precipitated urease in dilutions up to 600,000. Urease which had been denatured by contact with 0.05 N hydrochloric acid for a few seconds and which was then brought to neutrality gave no precipitate with antiurease whatsoever. Urease completely inactivated by formalin was non-toxic and produced no antiurease when injected into rabbits.

It was shown that antiurease protected animals from poisoning with urease. When two rabbits were given 90 neutralization units of antiurease each and three hours later 90 units of urease were given there were no symptoms of poisoning. A rabbit given 90 units of antiurease just before being given an injection of 80 units of urease was likewise unaffected. On the contrary, two rabbits which were given injections of 90 units and one rabbit given an injection containing 80 units of urease all died within five hours. All injections were intraperitoneal except the third rabbit which received the antiurease by ear vein.

In order to find out whether rabbits near death from urease poisoning could be saved by injecting antiurease, six two-kilogram rabbits were injected intraperitoneally with 65 to 70 units of urease. When the rabbits had become totally paralyzed (1½ to two hours afterwards) each rabbit was given 80 units of antiurease by injection into the ear vein. Four of the rabbits showed immediate improvement and became normal within one hour. The other two died. Similar results were obtained with guinea pigs.

*Antibody against Trypsin, Chymotrypsin and Chymotrypsinogen.* The methods of preparation and chemical properties of some of the crystalline enzymes as found by the Rockefeller group at Princeton are compiled in a book by Northrop, *et al.* (1948). Since it was demonstrated that chymotrypsin differs in its properties from trypsin, Ten Broeck (1934) undertook the demonstration of an immunological difference between the two enzymes. Trypsin and chymotrypsin are the enzymes principally responsible for the proteinase activity of pancreatic juice. Neither alone digests protein very far, but the two together cause hydrolysis to proceed to the polypeptide stage. Trypsin decreases the clotting time of normal or hemophilic blood, but under

ordinary conditions it does not clot milk. Chymotrypsin, on the other hand, clots milk but not blood. It probably represents the pancreatic rennin of Vernon. Trypsin appears to be identical in its specificity with Waldschmidt-Leitz's "Proteinase."

*Sensitization of Guinea Pigs.* The antibody production by the above enzymes and pro-enzymes was tested by anaphylactic test. This test, rather than the less sensitive precipitin reaction, was used in the major portion of the tests for differentiation for the reason that the enzymes may be very closely related. Since the sensitization of an animal is tantamount to immunization and anaphylactic shock depends upon an antigen-antibody reaction, the anaphylactic reactions have the same significance for the question under consideration as the precipitin reaction *in vitro* (Landsteiner, 1936). Female guinea pigs weighing about 125 g. were given subcutaneous injections of 0.5 ml. of either 0.5 or 1 per cent solution of the enzymes, purified by five crystallizations. Injections of chymotrypsinogen produced no visible effect, but the animals receiving the trypsin and chymotrypsin showed necrotic areas at the site of inoculation, and several of them died. Chymotrypsin seemed to be more toxic than trypsin. Between 15 and 20 days after the first injection the uteri of these guinea pigs were tested by means of the Dale technique (Dale, 1931). Two baths were used and the two horns of the uterus were tested, one after the other. The capacity of each bath was 75 ml. and 0.75 mg. of the enzyme solution was added for each test (1:1000 dilution of the enzyme in the bath). The uteri of guinea pigs receiving pig trypsin injections did not react against beef trypsin and chymotrypsin in 1:1000 concentrations; on the other hand, pig trypsin showed definite positive reactions with both left and right horns of the guinea pig uterus sensitized with pig trypsin. In three groups of similar tests the results showed that (1) guinea pigs receiving beef trypsin were sensitized against itself, but not against chymotrypsin and chymotrypsinogen; (2) guinea pigs receiving chymotrypsinogen were sensitized against itself but not against chymotrypsin and beef trypsin; and (3) guinea pigs receiving chymotrypsin were sensitized against itself, but not against chymotrypsinogen. Ten Broeck makes the following statement as a summary of his studies: "Not all of the animals were sensitized, and in some cases there were cross-reactions, particularly between the chymotrypsin and chymotrypsinogen. The results were, however,

sufficiently clear cut to show that all four of these enzymes and their precursors can be differentiated by this reaction."

*Antibody against Pepsin and Pepsinogen.* Seastone and Herriott (1937) carried out experiments to distinguish by serological methods the pepsins from several different animal species as well as to compare the serological behavior of pepsin and its precursor, pepsinogen. Northrop had previously reported (1930) that crystalline swine pepsin protein gave rise to pepsin precipitating antibodies. Seastone and Herriott were aware of the fact commented on by other investigators, that pepsin is inactivated above pH 6; as a more alkaline condition is approached the enzyme is converted into a typical denatured protein. At normal body temperature and at blood pH 7.6 it is therefore most likely that active pepsin in the body fluids is inactive, and denatured pepsin is responsible for antibodies developed following the injection of active pepsin. The limitations imposed by its denaturation at pH 7.6 must be accepted.

*Immunization.* Rabbits weighing about 2 kg. were given, at weekly intervals, three intraperitoneal injections of 5.0 ml. of a 1 per cent solution of swine pepsin at pH 5.0. This material had been twice crystallized and dialyzed. Two weeks after the last injection, the serum was collected. Precipitin reactions were done by the ring test, and readings were made after 1½ hours at room temperature. Of four rabbit sera, two showed no pepsin precipitins; one precipitated pepsin solution (pH 7.6) at a concentration of 1:1000 and 1:100,000. Anti-sera against swine serum were also prepared, the strongest reacting with normal swine sera in dilutions (on the basis of dry weight) of 1:100,000. Pepsinogen gave rise to precipitating antibodies more readily than pepsin. At pH 7.6 it is a stable native protein. Of the four rabbits immunized with pepsinogen, two had a titer of 1:100,000 and two 1:1,000,000.

Their findings further showed that alkali (pH 7.6) denatured pepsins from swine, cattle, and guinea pigs precipitated in swine pepsin antiserum; pepsin from the rabbit, chicken, and shark treated similarly did not precipitate in swine pepsin antiserum. Pepsin antisera reacted with both pepsin and pepsinogen but did not react with the serum proteins from the homologous species. Anti-sera made with serum proteins did not react with the homologous pepsin or pepsinogen. Pepsinogen anti-sera reacted with pepsinogen, but not with

twice-crystallized pepsin, nor with the serum proteins from the homologous species.

These findings demonstrate beyond doubt that pepsin protein is specifically antigenic. It also appears that pepsin retains the serological specificity of pepsinogen to an appreciable degree. On the other hand, pepsinogen is serologically an independent entity. Its activation into pepsin, associated with chemical changes (Herriott) partially retains this specificity.

*Antibody against Catalase.* After the isolation of beef liver catalase by Sumner and Dounce (1937) and horse liver catalase by Dounce and Frampton (1939) in crystalline form, Tria (1939), and Campbell and Fourt (1939) reported the immunization of rabbits with crystalline beef catalase. Tria immunized rabbits by injecting them with 12.5 mg. of enzyme every three days for three weeks in the first set of experiments. In a second set of experiments he started with 1.25 mg. of enzyme and gave gradually increasing doses. The immune sera had high anti-catalase activity. The sera reacted in 1:10 optimal dilution with a solution of catalase containing 0.1 to 1 mg. Determining the anti-catalase activity of the serum quantitatively he obtained 4 anti-catalase units in 1 ml. serum. A 50-fold purified anti-catalase isolated from the catalase-anti-catalase precipitate after dialysis had 2200 anti-catalase units per g. of antibody.

Anaphylactic experiments by Tria also showed the presence of antibody in the serum of guinea pigs actively immunized against beef and horse liver catalase. Anaphylactic shock resulted in the death of the immune animals within a few minutes.

The results of anaphylactic experiments aiming at a serological differentiation of the species specificity of beef, lamb, and horse liver catalases were inconclusive. The precipitation tests by Campbell and Fourt likewise demonstrated the formation of antibody against beef liver catalase. The results obtained from experiments involving the use of dog and horse liver catalases as antigens likewise did not yield conclusive information regarding the question of the species specificity of catalases.

**Summary and Conclusions.** The formation of antibodies in the animal system in response to antigenic stimuli has been discussed from various viewpoints. The stoichiometrical relationship underlies all known chemical changes—simple combination, decomposition, double



decomposition, tautomerism and mutarotation. A catalyst does not change stoichiometrical relationships of reactions, does not enter into any irreversible stable union either with the reactants or with the reaction products; its function is therefore one of repetition and continuity. For this reason it can transform a disproportionately large amount of substrates into reaction products. Numerous examples have been cited to emphasize this property of inorganic and organic catalysts (enzymes). Antigens likewise have been shown to produce proportionally large amounts of antibodies. Catalysts and enzymes in no case have been shown to be part of the reaction products; similarly, highly sensitive qualitative and quantitative analytical tests have not been able to demonstrate the presence of antigens or their parts ("marked" antigens) in the antibody molecules. It thus appears that antigens do not function as reactants in the stoichiometrical sense in the formation or synthesis of antibody molecules. The experimental data which have been presented would therefore seem to show that the rôle of antigens in stimulating the formation of specific antibodies is one of catalysis, which fact brings the antigens within the class of biocatalysts.

If antigens are believed to exercise a catalytic rôle in the production of antibodies, enzymes likewise have been shown to demonstrate the property of stimulating the production of specific antibodies. This property of enzyme proteins has been a subject of controversy for many years, although recently it has been accepted as an established fact. Thus antigens and enzymes possess two properties in common—catalytic activity and antibody production. These two properties are interwoven in the production of antibodies.

#### 4. Do Catalysts (Antigens) Make a New Reaction Possible?

The two properties of antigens and catalysts discussed above meet two of the basic criteria of the concept of catalysis. These properties are: (a) infinitesimal amounts of inorganic catalysts, enzymes and antigens catalyze the interaction of disproportionately large amount of reactants, and (b) neither antigens nor enzymes (and inorganic catalysts) form a part of the reaction products. There is also another criterion of the concept of catalysis which must be satisfied by enzymes and catalysts as well as antigens. A catalyst can only accelerate (not cause) a thermodynamically possible reaction. In other words, a catalyst

does not create a new reaction; it does not make the impossible possible; it simply accelerates a possible reaction.

It has been stated that "a catalyst not only accelerates a reaction, but makes a reaction possible" (Willstätter, 1927; Schade, 1923; see also Mittasch, 1935, 1938). This statement should not be taken too literally. This implies, for example, that hydrolysis of proteins is not demonstrable in the absence of proteolytic enzymes, but in the presence of traces of these agents marked hydrolysis occurs. The effect is dramatic. At the surface it may appear that such an effect is equivalent to the creation of new substances by, apparently, non-existent reactions. These processes are, however, thermodynamically possible. The fact that a catalyst encourages and accelerates such tendencies which already theoretically exist is not equivalent to the creation of a new reaction, or making the impossible possible.

## 5. Does Antibody Synthesis Involve New Processes Which Did Not Already Exist in the Animal System?

If we assume that the basic chemical processes responsible for the synthesis of antibody are different from those of the serum globulins we must also assume and demonstrate that there are basic chemical differences between the antibody and serum globulins. If this should prove to be true, then it could also be assumed that the antigen has produced in the animal system new processes for the synthesis of antibodies. This would necessarily create a discrepancy between the concept of catalysis and that of antigen exercising the rôle of a catalyst. If on the other hand, the available experimental data show that the chemical characteristics of both the antibody and serum globulins are practically indistinguishable then it is reasonable to accept the thesis that the synthetic processes involved for both the immune and normal globulins are essentially the same. The directive influences, however, may bring about certain configurational changes in the globulin molecules to account for the serological specificity of antibody globulins. In other words, the change from normal globulin to antibody globulin involves a change of the "configurational pattern" and not of the basic structures. Thus the above mentioned discrepancy between the concept of catalysis and that of antigen acting as a catalyst would not exist. This point of view will receive further support if we take



into consideration the occurrence of iso-antibodies as precursors or prototypes of all the antibodies.

*Iso-Antibodies.* From the known facts it can be concluded that the unique influence of antigens apparently is the shaping of certain parts or groups of normal globulins in conformity with the specific parts of antigens. Even this influence does not seem to be a new process of animal cells. The presence of iso-antibodies in the animal systems shows that antibody synthesis is a genetically established process. The formation of additional specific antibodies in response to the injected bacterial and other foreign proteins appears, therefore, to be an extension of the number and art of the synthesis of antibody globulins already being manufactured; in other words, a change of certain details in the genetically determined general scheme of antibody and serum globulin synthesis.

Landsteiner (1900, 1901) described and established the occurrence of iso-antibodies and their corresponding agglutinogens in human blood. He divided the blood from normal individuals which he examined, into three groups, namely: A, B, and C, on the basis of the agglutinative reactions. The sera of group A agglutinated the red corpuscles of group B, but not of group C; the sera of group B agglutinated the red corpuscles of A but not of group C; the sera of group C agglutinated the corpuscles of both A and B. The sera of the fourth group described by Decastello and Sturli (1902) failed to agglutinate the corpuscles of the above cited three groups, but its corpuscles were agglutinated by the sera of all of them. The blood of zoologically related species shows immunological relationship; thus the bloods of chimpanzee and man, mouse and rat, etc. are related. The blood cells of pigeon, rabbit and man are agglutinated by goat's serum; and the blood cells of each species will absorb out from goat's serum its own specific agglutinin but not the agglutinin which reacts with the blood cells of the other species.

The four classical blood groups are inherited according to Mendelian principles, and all experimental evidence to date points towards the chromosomes as the bearers of the hereditary factors, or "genes." What interests us here most directly is the fact that the synthesis of these antibody globulins is a genetically determined process.

The basic antigenic unit in natural and artificial conjugated proteins is the natural protein itself. The prosthetic groups coupled with

the protein do not initiate the formation of, but contribute to the architectural pattern (specificity) of the antibodies. It is immaterial whether a protein is used in its natural form or after having been coupled with a chemical group non-existent in nature. The coupled prosthetic groups which merely influence the specificity of antibodies are comparable to the type or group specific carbohydrates of various bacterial antigens. The prosthetic groups of artificial or natural conjugated antigens in many respects are similar to the conjugated enzyme proteins. In conjugated protein antigens the prosthetic groups alone are non-antigenic; similarly the prosthetic groups of the conjugated protein enzymes are catalytically inactive by themselves. The heme group in hemoglobin, catalase and peroxidase is comparatively inactive without a combination with their specific native proteins. Various co-enzymes must likewise be in conjugation with specific proteins to manifest their activity.



## Part II

# Mechanism of Antibody Formation

### A. THE FACTORS CONTROLLING THE PRODUCTION AND PERSISTENCE OF ANTIBODY

**A**PRESENTATION of a chain of reactions leading to the appearance of immune bodies in response to an antigenic stimulus in the complex *in vivo* environment is obviously impossible because of present lack of precise information. It is likewise difficult to ascertain the nature of the factors which influence the rate of formation, rise and decline in the amount of, and eventual disappearance of antibody from humoral systems. An approximation to these questions may, perhaps, be achieved by an analysis of the available results which may have bearing on these questions. The following discussion is an attempt in this direction.

#### 1. The Relation of the Specificities of Host Enzymes to the Antigenicity of Substances Foreign to the Species of the Host

In connection with the problems pertaining to immune response to an antigenic stimulus, there are two questions into which we need to inquire. They are:

- a. The absence of an immune response to an antigenic substance derived from one individual in another belonging to the same species;\* and

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\*There are reported certain results which might appear to be not in full agreement with the view here discussed. For example, Hektoen and Schulhof (1925) reported that a rabbit *thyroglobulin* produced a precipitin in a rabbit. Also overlapping precipitin reactions were obtained among thyroglobulins of beef, dog, horse and human; human and rat; horse, human and sheep; beef, sheep and human, and swine, human, sheep and dog. On the other hand, Stokinger and Heidelberger (1937) demonstrated definite species differences among various thyroglobulins in addition to organ specificity, corresponding in general to the biological relationship of the animals from

- b. The immediate immune response to an antigenic substance when introduced into an individual belonging to a different species.

A reasonable understanding of the difference in immune response under the above two conditions would necessitate that we become acquainted with *in vivo* metabolism of protein and other non-protein antigenic substances. It is an established fact that antibody protein is a modified globulin. Immune globulin which has been obtained from

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which the thyroglobulins were derived. Thyroglobulin is a conjugated protein containing thyroxine and a protein. Since the thyroxine group, common to the thyroglobulins of various species, would be expected *not* to function as a common determinant haptenic group, the overlapping precipitin reactions described by Hektoen and Schulhof could be due to the denaturation and thereby loss of the specificity of thyroglobulins they isolated. Thyroglobulin is known to circulate in normal blood and does not function as an antigen under these circumstances. Its reported antigenicity in homologous species might very well be due to its denaturation incurred during its preparation.

Kato (1924) reported that the serum of rabbits immunized with a heterologous *fibrinogen* may react with it and also with that of other species, but not with rabbit fibrinogen; and the serum of rabbits immunized with rabbit fibrinogen reacts with other fibrinogens but not with the immunizing rabbit fibrinogen. These findings would indicate that the rabbit fibrinogen exercises species specific and non-specific serological activity as well, and that fibrinogens from various species possess a common denominator or, as antigens, they represent mixtures of denatured and native fibrinogens. Hektoen and Welker (1927) obtained overlapping reactions among the fibrinogens from chicken, duck, goose, guinea-hen, pigeon and turkey. They studied the fibrinogens of the common mammals and concluded that they have antigenic elements that are more or less common, but that bird fibrinogen does not belong immunologically to this group; however, it is not wholly distinct and different from mammalian fibrinogen. Fibrinogen is unique among the serum proteins. It is insoluble in salt-free water but is soluble in dilute salt solutions. It is the most readily precipitable of all the common blood proteins by concentrated salt solutions e.g., half saturation with sodium chloride or 20 per cent ammonium sulfate. It is also unique among the blood proteins in that it is readily converted into insoluble fibrin by the action of thrombin. It is an asymmetrical molecule with dimensions of  $33 \times 900 \text{ \AA}$ . Though the coagulation temperature of fibrinogen is  $55^\circ\text{C}$  in neutral solution, the heat coagulation of fibrinogen (and fibrin) during a period of one hour, according to Robbins (1945), did not affect the antigenic nucleus.

In view of these properties, one may wonder whether or not the laboratory preparations of fibrinogen are removed from the native form. In this respect it may be of some interest to reinvestigate the problem with a view to the role of  $-S-S-$  or  $-SH$  groups in fibrinogen in relation to their possibly masked immunological specificities as has been demonstrated with keratins and ocular lens proteins discussed below, also for the possible reasons that, according to Baumberger's suggestion (1941),  $-S-S- \rightleftharpoons -SH$  relationship might play a role in the conversion of fibrinogen into fibrin, and that reducing agents, e.g., cysteine, glutathione, sodium bisulfite (Chargaff, 1945) inhibit fibrin formation (see page 282).

For a long time keratins were considered immunologically indistinguishable. Pillemer, Ecker and Wells (1939), Pillemer, Ecker and Martienssen (1939) showed, however, that species specificity is an individual characteristic of *keratins* and that the observed specificity is dependent on the redox state of the sulfhydryl groupings in the protein molecule. In a similar study, Ecker and Pillemer (1940), contrary to



the antigen-antibody complex and purified by physico-chemical means, has been shown to possess the serological species-specificity of normal globulin and the acquired additional property of reacting specifically with the homologous antigen. It would be of interest, therefore, to consider the metabolism of normal and immune globulins, and other proteins as well. Experimenting with young and adult rabbits, Cannon (1945), Cannon, *et al.* (1943), and Wissler, *et al.* (1946) reported

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the long accepted indistinguishable specificity of the *proteins of the ocular lens*, demonstrated the species specificity of the proteins of lens from chicken and fish, though this characteristic was not so evident in the closely related species swine and sheep.

According to Lewis (1934) *casein* is iso-antigenic. Sera of two goats, one immunized with goat casein, and the other with cow casein, reacted equally well with both caseins. The chief objection to these results seems to be the method of the preparation of casein which involved precipitation with normal acids and mechanical stirring at the rate of 2000 to 3000 revolutions per minute. The system was exposed to 0.1N acid (final concentration) for six to eight hours. The precipitated casein was washed five times with water, twice with 95 per cent alcohol and three times with ether. It was then dried by spreading. Under these conditions the species specificity of native casein might easily be destroyed as a result of denaturation, and behave like a non-specific antigen. For it has been shown that, in contrast to native rabbit serum protein, denatured rabbit serum would produce antibody in the rabbit (Landsteiner, 1945).

One may also call to attention the possible non-specific antigenicity of the prosthetic group, serine-phosphate or glutamyl-serine phosphate (Levene and Hill, 1933; Schmidt, 1933, 1934), common to all mammalian caseins. The phosphate in casein is ortho-phosphoric acid esterified with the hydroxyl group of serine. It is a relatively strong acid and its titration curve shows a sharp inflection between pH 6.0 and 7.5, due to, possibly, secondary ionization of the phosphoric acid group. The antigenicity of phenyl phosphoric acid is an established serological fact.

Another possibility which may be worth mentioning is the fact that casein is a protein synthesized by the female mammal by an organ which is entirely inactive and undeveloped in the male. For these reasons one may be tempted to consider it as a protein foreign to any mammalian system. Bruynoghe (1935) reported that the *egg-white* from a hen, precipitated with ammonium sulfate and dialyzed to remove the salt, produced specific antibody in the same hen and in a rooster. He assumed that egg-albumin is a protein totally different from the components of serum and has not participated at all in the cellular metabolism of the animal. In this manner, he concluded, egg-albumin might exercise iso-antigenic activity. However, one must keep in mind the fact that egg-albumin is very susceptible to denaturation during its preparation.

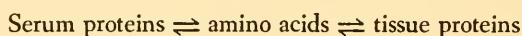
Ferritin is a metal protein of 500,000 molecular weight and contains from 17 per cent to 23 per cent Fe. Granick (1943) found that apoferritin derived from crystalline ferritin, with the exception of a slight reaction between horse apoferritin antibody and dog apoferritin, is immunologically species specific, and non-organ specific. Protein crystals of ferritin obtained from one organ reacted with antiserum to ferritin crystals obtained from another organ.

Bonnichsen (1947) found that liver and blood crystalline catalases were serologically identical; the values for nitrogen distribution, histidine, arginine, lysine, tyrosine, cystine, glutamic and aspartic acids were found to agree within the limits of error for both catalases.



that "Adult rabbits made hypoproteinemic by a low protein diet or by low-protein diet supplemented by plasmapheresis exhibited a lessened capacity to produce agglutinins as compared with animals of similar age but supplied with an adequate diet," and "Protein repletion of protein-depleted rats by the feeding of high-quality protein, or a hydrolyzate of a high quality protein led to a markedly increased output of hemolysin, evident as early as after two days of repletion and pronounced within seven days." The agglutinative titers revealed that the average agglutinin-output of the well-fed rabbits was about five times that of the protein-depleted ones. The serum protein levels of the well-fed group rose to an average of 6.62 grams per 100 ml. whereas those of the low-protein group of rabbits declined to an average level of 4.76. These results show that factors controlling protein metabolism are directly related to the production of antibody.

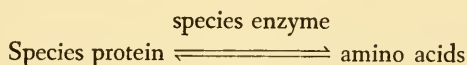
According to the studies of Whipple and his collaborators there exists a dynamic equilibrium between the proteins of blood and those of tissues. That is, there is a "give and take" between body and plasma proteins. When plasma protein is depleted, replacement is possible by the proteins of the organs (Madden and Whipple, 1940). The results of the studies of Schoenheimer and his collaborators (1942) with isotopic amino acids have shown that the proteins are in dynamic equilibrium with their constituent fragments. When an isotopic amino acid was added to the diet of a host, the concentration of marked nitrogen in the serum proteins increased immediately, but diminished steadily after the addition of isotopic amino acid to the diet was discontinued. Chemical reactions had thus occurred among the units of the individual serum proteins, resulting in the uptake of dietary nitrogen in the first period and its removal in the second. The above findings may be presented in the following manner:



This dynamic equilibrium of protein metabolism, involving proteolysis and protein synthesis is mediated by species specific proteinases. From what we know of the species-specificity of proteins and the specificity of enzymes, it would be reasonable to assume that the enzymes of the individuals belonging to the same species metabolize each other's proteins, given parenterally, in an identical manner. Under these con-

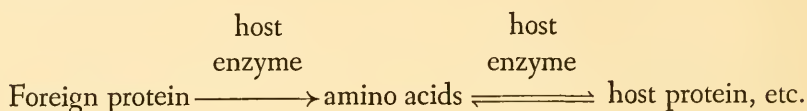
ditions, they can exercise no antigenic stimuli. The readiness with which these proteins are dispensed with is most likely due to an absence of structural difference among the proteins of the individuals of a given species. In contrast, the parenteral injection of a protein, derived from a species different from the species of the recipient host, being structurally different will resist greatly the action of the host enzymes. Due to this resistance, the life of the whole protein, or its structurally specific part, will be prolonged. Transferred by phagocytes to a center of protein metabolism, not only will it resist complete degradation but would seem also to influence the course of the synthesis and certain details in the pattern of the normal globulin, yielding immune antibody globulin. In this manner, the foreign protein, or antigenic unit, exercises the role of specific catalytic modifier, or superimposes a new catalytic role of its own on the enzymes which synthesize globulins. This role continues so long as the antigenic unit remains intact. Any process whereby the life of the antigen in the host is prolonged might result in a degree of antibody response.

The difference between a species specific substance and that which is foreign to the host rests on the distinctive differences in the specificities of host enzymes (or the genes which are assumed to be responsible for the origin of enzymes). The ready digestibility and utilization of a species protein by the species specific host enzyme system is understandable, for the specialized enzyme system is capable not only of digesting such a protein when parenterally introduced but also is capable of synthesizing it in an identical pattern. This may be presented in the following manner:



On the other hand, a host can either eliminate a foreign protein (or substance) by excretion, or digestion by virtue of the inherent abilities of proteolytic enzymes to split peptide linkages common to all proteins. Because of the basic structural (or architectural) difference of the molecular species, there is no doubt that the elimination of the foreign protein when parenterally introduced, will proceed at a very different rate than the digestion of the species specific protein. In contrast to this ability of the host enzymes to eliminate a foreign substance, they totally lack the ability to resynthesize or regenerate the foreign-

protein from its hydrolytic products. This would mean that the host enzyme would catalyze the reactions of a foreign protein *only in a forward direction*:



This relationship is particularly worthy of consideration when we are dealing with artificial conjugated antigens. These differences in the rates of the metabolism of species specific and foreign proteins, or the failure to metabolize a foreign protein by a host could no doubt account for the longer life of a foreign substance in a host as will be discussed below. This relationship is of fundamental significance in the production of antibodies to foreign substances.

A foreign protein may possess component units uncommon to the host species. Many microorganisms possess unusual proteins or polypeptides composed of single or different unnatural amino acids, and non-protein uncommon substances as well. Ivánovics and Bruckner (1937, 1940) found that the specifically precipitable capsular substance of *B. anthracis* is a polypeptide-like substance of a molecular weight of about 6000 made up solely of 40 to 50 d-glutamic acid residues. According to Hanby and Rydon (1946) the capsular substance is made up solely from d-glutamic acid residues. The molecular weight of the native material is greater than 50,000 and is thus of the same order of size as many proteins. Structurally, the capsular substance is a long chain molecule made up of  $\alpha$ -peptide chains of 50 to 100 d-glutamic acid residues joined together by  $\gamma$ -peptide chains of d-glutamic acid residues. Since d-glutamic acid is of unnatural optical configuration the proteolytic enzymes of the animal system would either fail to digest or effect the digestion of this polypeptide at a very slow rate. The resistance of this polypeptide to proteolysis would not only confer on it a haptenic role but may also serve as an armor for the microorganism against the host enzymes, prolonging the antigenic activity of the cellular components and virulence as well. The occurrence of polypeptides which are indistinguishable from that of the anthrax bacillus have been likewise found in the organisms of the mesentericus and subtilis groups. In *Saccharomyces cerevisiae* a polypeptide composed of 10 to 12 glutamic acid residues was found to be

linked at the terminal part of the chain to one molecule of p-amino-benzoic acid through a carboxyl group, as reported by Ratner, *et al.* (1944). It is also to be noted that gramicidin and tyrothricin obtained from *B. brevis* are polypeptide containing natural l- and unnatural d-amino acids. These polypeptides are resistant to the action of crude trypsin, pepsin, papain and papaya latex at several pH values (Hotchkiss, 1944). This resistance is attributed to the d-amino acid contents of these polypeptides which exercise antibacterial action and toxicity for animal cells and tissues.

The consideration of the above cited observations may help us to visualize the structural differences of antigenic substances derived from animal, plant and bacterial sources. These differences, no doubt, play a significant role in resisting the host enzymes and thereby prolonging the life of antigenic substances and the immune response they produce in a host. In this connection, the following observations are of interest. In a study on the behavior of antibody protein toward dietary nitrogen in active and passive immunization, the following experiment has been carried out (Heidelberger, *et al.*; Schoenheimer, *et al.* 1942). A rabbit actively immunized against Type III pneumococcus was given a single large injection of Type I antipneumococcal rabbit serum. The administration of isotopic glycine by addition to the stock diet was started two hours before injection and continued for 48 hours. Daily estimations of the amount of circulating antibody (passively administered) and of the isotopic N<sup>15</sup> concentration of antibody and residual proteins were made. Since, in this passive immunization, the antibody introduced could only be hydrolyzed but not be regenerated, its daily estimation gave an idea as to how long it could persist in the rabbit acting as a species specific host. At 0 hour the amount of passively introduced Type I antibody corresponded to 1.09 mg. of total antibody nitrogen/ml. of serum. After 22½ hours it was 0.88 and after 48 hours 0.49 mg. of total antibody nitrogen/ml. of serum. Thus 55 per cent of the passively introduced antibody was eliminated within 48 hours. Attention was drawn to the fact that the passively introduced antibody enters into metabolic reactions which lead to its disappearance, but not to the regenerative uptake of nitrogen, as in the case of actively produced antibody. The failure of passively introduced antibody to take up heavy nitrogen, they stated, can less reasonably be ascribed to a generalized "foreign protein" character than to the absence of some



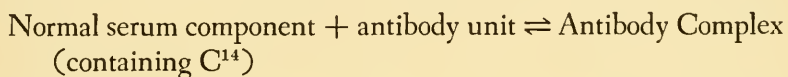
specific function of the tissue cells of the host, meaning the specific function which is operative in active immunity.

The rate at which the passively introduced anti-pneumococcal rabbit antibody disappears in the body of the rabbit shows that 37.6 per cent was eliminated during 22½ hours, and 55 per cent during 48 hours. In contrast, the foreign proteins (or polysaccharides) administered to a host for immunization or for therapeutic reasons persist for a decidedly longer period of time. As will be discussed below, production of an antibody can occur within four to 10 hours after the injection of an antigenic material. It is therefore reasonable to assume that the length of the period, during which a species specific protein can persist in a host of the same species, appears to be long enough to exercise an antigenic stimulus. The absence of such a stimulus would emphasize the importance of the difference of molecular structure of the foreign antigenic substances from those of the host as the most critical difference in the stimulation of an immune response.

In contradiction to the observation of Heidelberger, *et al.*, Kooyman and Campbell (1948) reported that antibody globulin introduced passively into rabbits can enter into the dynamic equilibrium state of the body without loss of antibody properties. Experiments were carried out by injecting C<sup>14</sup> labeled dl-leucine into a rabbit which was actively immunized against p-azophenylarsonic acid-ovalbumin. Ten days after the last injection, an injection of 10 ml. of concentrated rabbit pneumococcus antiserum (Type I) was given intravenously, followed by a similar injection next day. On the same day, an injection of 30 ml. of a one per cent leucine solution was given intraperitoneally. The leucine contained C<sup>14</sup> in the carboxyl group. Similar injections of leucine were given on the three following days, 1.15 g. of leucine being injected in all. Samples of serum were taken on 5, 9, and 16 days after. The antibodies were precipitated with corresponding antigens, washed with saline three times, once with distilled water, twice with alcohol and ether, and analyzed for C<sup>14</sup> after drying. These materials were reported to contain labeled carbon.

If interpreted to indicate that passively introduced antibody globulin is digested to the stage of amino acids and peptides and the C<sup>14</sup> containing antibody globulin is resynthesized in the absence of the specific antigen, these findings would be contrary to the facts underlying the concept of the mechanism of antibody formation, namely, that antibody

synthesis occurs under the specific stimulus of an antigen. No exception to this rule has as yet been found. The presence of  $C^{14}$  in the assayed antigen-antibody precipitates could, perhaps, be best explained in the following manner: (a) Antibody globulin molecule can exchange amino acid residues in a dynamic environment without undergoing appreciable digestion or losing parts from the basic antibody molecular unit; (b) normal serum components and the minimum antibody molecular units (e.g., diphtheria antitoxin, etc.) can enter into reversible combinations, as shown below:



and (c) the extreme precaution taken to remove possible  $C^{14}$  impurities from the antigen-antibody precipitates was perhaps inadequate and drastic dialysis at various  $[H^+]$  might have been necessary to remove the  $C^{14}$  impurities from the reactants to start with.

With this in mind, the following observations concerning the length of time an antigenic substance can reside in a host are presented. According to Uhlenhuth and Weidanz (1909) 5 ml. of horse serum injected intravenously into a rabbit was found in the blood 15 days after the injection. With the gradual increase of precipitin there was a corresponding diminution of the horse serum. Longcope and Mackenzie (1920) reported that 5 ml. of horse serum per kilo body weight of rabbit injected intravenously was detectable after three weeks. These latter investigators studied also the presence of horse serum at given intervals after its injection into human beings for therapy of pneumonia. The results of experiments with 14 individuals, who received from 100 to 500 ml. of antipneumococcal horse serum intravenously yielded results falling into two groups.

In the first group of eight cases, the persistence of horse serum ranged from 18 to 39 days. The injection of horse serum was followed by severe serum disease, lasting from 11 to 28 days, and as a rule the precipitins appeared first or their concentration increased markedly towards the end of the serum disease. On the other hand, the precipitin reaction for horse serum diminished rapidly towards the termination of the serum disease and disappeared shortly thereafter. In the second group of 5 cases, the precipitinogen in the serum persisted from 49 to 69 days. The precipitin formation was either of extremely short



duration or entirely absent. These patients either had very mild serum disease lasting from one to five days or had none at all. One individual who had received 630 ml. of horse serum showed the persistence of horse serum in the circulation for 75 days. He also showed precipitins and had severe serum disease lasting 12 days. These observations were made, in the main, by the use of macroscopic precipitin tests, and therefore are rough estimations. By the use of refined quantitative microtechnique one may be able to detect circulating antigens during a longer period of time. Such estimations, however, would fail to inform us about the amount and the duration of antigens bound in the tissues which may continue to exercise antigenic stimuli during a far greater period of time than are indicated by the tests for their presence in blood and urine.

Herdegen, Halbert and Mudd (1947) developed an *in vivo* technique whereby they demonstrated that 0.002 mg. of *Shigella paradyenteriae* Type III antigen was capable of inducing an agglutinin response. This method was used to determine the fate of this antigen in mineral oil emulsion stabilized with lanolin derivatives at the site of subcutaneous inoculation in mice. It was found that the antigen persisted at the site of injection for at least 24 weeks when 200  $\mu$ g. of antigen were injected. This was demonstrated by covering the injected vaccine from period to period; 200  $\mu$ g. of antigen per 0.3 ml. of original volume of vaccine was recovered after a period of two weeks. This decreased to 20  $\mu$ g. after 12 weeks, but 0.2  $\mu$ g. was still present at 24 weeks. The material recovered from the injection site was still antigenic. The deterioration of antigen at the site of inoculation was paralleled by a fall in the agglutinin titers of the mice receiving the oil vaccine.

In connection with the above observations, a reference to the findings of Freund and Bonato (1946) is of interest. Using water-in-oil emulsion stabilized by Falba (a mixture of oxysterine and cholesterol derived from lanolin) of killed typhoid bacilli as vaccine, they reported the presence of antibodies in the sera of animals three years and one month after one injection and three years and five months after two simultaneous subcutaneous injections.

The persistence of pneumococcal polysaccharide in the circulation for a long period of time has been reported by various investigators. Dochez and Avery (1917) carried out a systematic study of the pres-

ence of soluble specific substance in the blood and urine of experimental animals and of patients suffering from lobar pneumonia. A positive reaction for soluble specific substances (Types I, II, III) in the blood was found as early as 12 hours after the initial chill, and was demonstrable in one instance five weeks after defervescence. A positive precipitin test with urine against anti-pneumococcal serum was, in certain cases of Type I pneumonia, demonstrable as late as 42 days after the infection; with Type II pneumonia, after 58 days, and with Type III, 30 days after the infection. Quigley (1918) studied 82 cases of Types I, II, and III, pneumococcus lobar pneumonia and obtained positive urine tests for soluble specific substance in 81 per cent of the cases as late as the 21st day during convalescence. Similar results were obtained by Blake (1918). Pepper (1934) following the excretion of polysaccharide in urine by several cases of lobar pneumonia, reported that two Type I cases of empyema excreted large amounts of S substance late in the disease, one of them until the 41st day and the other until the 27th day. Urinary S substance did not appear until agglutinins for Type I pneumococci appeared in the blood.

In immunization experiments, Avery and Goebel (1933) found that acetyl polysaccharide, corresponding to the above cited soluble specific substance, persisted in the circulation of the treated rabbits for considerable period of time, was slowly excreted by the kidney, and appeared in the urine in its naturally acetylated form, though, for reasons as yet not understood, acetyl polysaccharide does not induce any immune response in the *rabbit*. However, the fact remains that the type specific antigenic component of pneumococcal vaccine, acetyl polysaccharide, is excreted without being attacked by the host enzymes. On the other hand, it is a well-established fact that this same substance, or its slightly modified forms, produce immunity in mice and men (Schiemann and Casper, 1927; Saito and Ulrich, 1928; Wadsworth and Brown, 1931; Zozaya and Clark, 1932, 1933; Sevag, 1934; Felton, 1935; Heidelberger *et al.*, 1946). It is further to be noted that the polysaccharides which persist in the circulation without producing immune response were shown to be antigenic determinants by coupling with serum globulin (Avery and Goebel, 1931). Type III polysaccharide coupled with serum globulin immunized rabbits against infection by virulent Type III pneumococcus, and the immune serum contained type specific antibodies which precipitated Type III

polysaccharide, agglutinated Type III pneumococci, and specifically protected mice against Type III infection. These findings show that these polysaccharides are, apparently, incapable of forming a polysaccharide-protein antigenic complex *in vivo* with rabbit proteins, in a manner, perhaps, comparable to those possibly formed when pure polysaccharides are injected into mice and men.

An interesting new observation on the effect of a large dose of pneumococcal carbohydrate on antibody production is reported by Felton, *et al.* (1947). A relatively large dose of antigenic polysaccharide of pneumococci type-specifically "paralyzes" the immunological mechanism of mice during the life span or for 15 months. This paralysis was reported to be due to the presence of polysaccharide. From observations of a large number of "paralyzed" mice, the pneumococcus polysaccharide was found present in the following tissues in order of decreasing precipitinogen concentration: liver, spleen, kidney, skin, bone marrow; irregularly present in muscle (especially Type III), lung, intestines, and urine, and absent in heart and blood. In three instances, the polysaccharide was isolated, partially purified, and tested for immunizing activity. Type I, 5 gamma protected against 50,000 lethal doses, Type II, 0.5 gamma against 500,000 lethal doses, and Type III, 5 gamma against 500 lethal doses. The amount of polysaccharide in the tissues gradually decreased with increasing interval following injection; but after 15 months, it was still present in the liver of animals paralyzed against Type I and II, and, in one experiment, Type III. In addition, mice injected with a paralyzing dose of pneumococcus whole-cell vaccine, showed a similar distribution of polysaccharide in the tissues. These observations may indicate, as the investigators suggested, that the large dose of polysaccharide "paralyzes" the antibody forming mechanism of the animal, or that antibody formed is neutralized by combining with polysaccharide and is immediately eliminated because of the continuous presence of a large excess of antigenic polysaccharide.

The immunological paralysis is a typical specific blocking, for antibodies to immunizing doses of heterologous antigens are synthesized. The enzyme systems which synthesize other immune globulins must therefore be in an intact state. Only the response to the polysaccharide antigen is lacking. Since the polysaccharide antigen is found in nearly all the cells of immunologically paralyzed animals, failure to demon-

strate the presence of homologous antibody was interpreted (Felton, 1949) to be due to so firm an attachment of the antigen to cell substances that it is no longer free to act as an antigen. If we accept the idea that antigen functions as a catalyst, the above interpretation of "immunological paralysis" would be in agreement with the fact that a catalyst which *irreversibly* combines with a component of a reaction system would be incapable of catalyzing the specific process.

The fact that these antigenic polysaccharides persist in the circulation for long periods and are excreted through the kidney unchanged fails to indicate a relationship between these results and an *in vitro* observation by Dubos and MacLeod (1938) that pneumococci are very rapidly rendered non-antigenic (in regard to type-specific antibody) by leucocyte extracts.

## 2. An Inquiry into the Nature of Factors Controlling the Balance of Antigen and Antibody During Immunization

For an understanding of the theories advanced concerning the mechanism of the formation of antibodies it may be of interest to consider the possible factors which influence the rate of antibody production, its decline, and also the length of the period during which an antigen may remain in the host system. The amount of antibody formed in response to antigenic stimuli will be dependent on the rate at which antibody is removed, or dissociates from the site of its synthesis into the humoral systems. With the accumulation of antibody in the humoral system and tissues an equilibrium condition will be established.\* The attainment of this final state of equilibrium may represent the peak of antibody concentration. The maintenance of the peak of antibody concentration would be dependent on several factors. The rate at which

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\*The question of how soon the above reaction equilibria can be set up *in vivo* may best be answered by a consideration of the following observations. Two hours after subcutaneous injection, egg-white was demonstrated in the urine and blood by the precipitin reaction (Ascoli, 1902; Obermayer and Pick, 1902). According to Dochez and Avery (1917), if rabbits are infected intraperitoneally with pneumococcus a substance specifically precipitable with antipneumococcus serum can be demonstrated in their blood stream, freed from bacteria by filtration, from within two to six hours following the time of infection. One may find other similar observations in the literature. These facts show how rapidly the antigenic material finds its way into the circulation introduced through various routes.



antibody is formed and the rate at which it is metabolized, antibody  $\rightleftharpoons$  amino acids  $\rightleftharpoons$  normal proteins, will condition the maintenance or decline of the peak of humoral concentration of antibody. The rate of antibody formation will be governed by three principal factors:

(a) The ability of antigen to resist the host's catabolic activities.

(b) The rate at which antibody accumulates and thereby shifts the equilibrium from left to right in the following complex reaction mechanism: cell-antigen-antibody  $\rightleftharpoons$  cell-antigen + antibody. Because of the strong specific affinity between antigen and antibody, antigen-antibody complex formation, in the presence of an accumulated amount of antibody, may predominate, blocking the activity of antigen (see p. 137) to direct the formation of new antibody.\* However, this condition cannot last long for the obvious reason that the amount of antibody will soon diminish by participating in the protein metabolism of the host, or by some special mechanism for eliminating antibody from the antibody forming cells, and, therefore, the reaction will resume its forward trend once again.

(c) The magnitude of the dissociation constants derivable from the reaction equilibria. The rates of the backward and forward reactions in the establishment of the states of equilibria are governed also by the magnitude of the dissociation constants of various reactions. The following may be cited to illustrate the possible reaction equilibria:

- (1) Antigen-cell  $\rightleftharpoons$  cell + antigen
- (2) Cell-antigen-antibody  $\rightleftharpoons$  cell + antigen + antibody
- (3) Antigen-cell-antibody  $\rightleftharpoons$  antigen + cell + antibody
- (4) Antigen-antibody  $\rightleftharpoons$  antigen + antibody

From the standpoint of continued antibody formation, the most critical of the above relationships would appear to be a very small degree of dissociation of the cell-antigen complex,<sup>†</sup> supplemented with

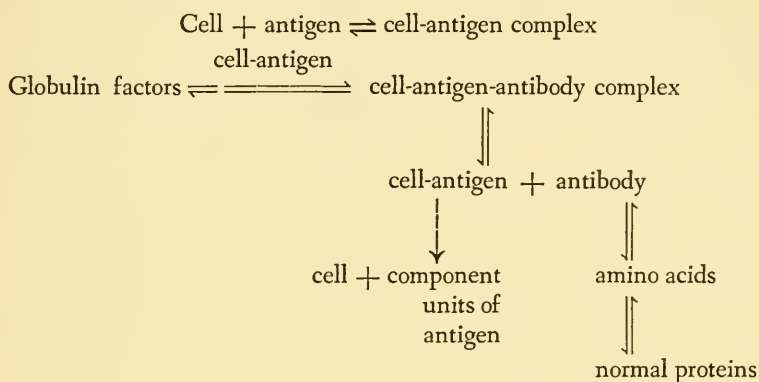
\*In the presence of an excess of antigen (severe infection) small amounts of antibody formed could not be demonstrated because of antigen-antibody complex formation and elimination.

<sup>†</sup>In connection with the above relationships it may be pointed out that the interaction between antigens, antibodies, and the susceptible tissue cells could be expected to exercise marked effects on the above reaction equilibria. It has been pointed out by Friedmann (1947), for example, that the avidity of tetanus toxin for the tissue cells will result in slower rate and degree of reaction between the toxin and the antitoxin. The combination between the tetanus toxin and tissue was stated to undergo a gradual increase in firmness and that, consequently, it became increasingly difficult for the antitoxin to dislodge the toxin from the tissue. The resistance of toxin to antitoxin under conditions as they prevail in the natural disease is explained by the high avidity



a greater degree of resistance of the antigenic molecule to the catabolic actions of the host enzymes.

The interrelationship of the various component parts of the complex reaction mechanism of antibody formation may, perhaps, be presented in the following scheme:



In the synthesis of antibody globulin, antigen functions as a catalyst when present in trace amounts. When present in large amounts it also functions as a reactant in a stoichiometrical reaction with antibody and thereby exercising a determinant rôle in regulating the amount of antibody procurable in the host. The above formulations would seem to be amply supported by the following observations.

According to Hamburger (1902), a precipitin reaction for egg-white appeared in the serum of rabbits two hours after subcutaneous injection, reached a maximum after 24 hours, persisted until the third day and had disappeared on the fourth day. Ramon (1928) reported that following the immunization of horses with the filtrates of formalized broth cultures of pest bacillus there was produced flocculating antibody within from six to eight hours after the injection. The serum obtained 36 hours after the injection contained a four-fold greater amount of antibody. Similar results were obtained in experiments with six other horses. The above cited observations may be looked upon as exceptional or unusual cases; however, they indicate the speed with which the immune mechanism may be set in operation following the introduction of antigens into a host.

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of the toxin for nerve tissues (see further Friedmann, 1947; Wilson and Miles, 1946).

That anti-virus antibodies enter into combinations with tissue cells has been shown by Sabin (1935) and Andrews (1928).

In an extensive investigation on the antigen-antibody balance in lobar pneumonia, Blake (1918) studied: (a) daily blood cultures throughout the course of the disease; (b) daily determinations of the concentration of soluble specific substance in the blood and urine, and (c) daily determinations of agglutinins and precipitins in the blood serum. All patients who failed to excrete soluble antigen in the urine during the course of the disease developed precipitins in the blood at or about the time of crisis, which may indicate that the development of precipitin in the body keeps pace with the elaboration of the antigen and that the two serve to neutralize each other until finally the development of precipitin exceeds the formation of soluble antigens, and free precipitin appears for the first time in the blood. Furthermore, the concentration of precipitin, after rising rapidly during the period shortly after crisis, fell rather abruptly coincident with the appearance of soluble antigen in the urine. Apparently, according to Blake, this is associated with the liberation of a considerable amount of antigen by the resolution of the pneumonic consolidation. At least it was coincident with it. The fact that in two cases during this period small amounts of precipitin in the blood and of soluble antigen in the urine were simultaneously present, was explained as due either to antigen and precipitin simultaneously present in approximately equal amounts in the body and not in all instances completely bound, or that the kidney possessed the power of separating the two, excreting the soluble antigen and retaining the precipitin. The fact also that recovery was invariably accompanied by the appearance of agglutinins was striking, and suggested to him that here again a struggle between living antigen (i.e., pneumococcus) and its corresponding antibody was taking place.

On the other hand, in those cases, usually severely infected, in which soluble antigen was excreted throughout the course of the disease, and in which precipitin did not appear in a free state in the blood, it appeared to Blake that the formation of precipitin never equalled the elaboration of soluble antigen, which was constantly in excess and readily excreted in the urine. He attributed his inability to detect the presence of soluble antigen in the blood in these cases to probable insufficient refinement of his method of testing. As will be discussed below, it may have been due to the constant neutralization and removal of the soluble antigen by the antibody present in the blood stream and excretion through the kidneys. It was also observed that, in

fatal cases, there was an increasing septicemia, steady rise in the amount of soluble antigen excreted in the urine, and complete failure to develop either precipitins or agglutinins in the blood. Apparently in fatal cases the body was unable to combat the progress of the infection by the production of sufficient antibodies to neutralize and overbalance either the living antigenic cell or its soluble products.

The coincidence of antigen and antibody in the blood of an immunized animal has been variously reported (for a brief review see Opie, 1923). Using whole serum or egg-white, Opie found that while an antigen can persist in a normal animal for a long time, it is more readily removed when introduced into an immune animal. The presence of antigen coincident with antibody shown in precipitin tests was associated with the use of complex antigenic mixtures such as whole serum. Apparently, differences in the relative amounts of antibodies formed to various antigens in such a mixture were responsible for these observations. When a repeatedly crystallized preparation of egg albumin was used as antigen its presence in the blood of a rabbit immunized (titer 1:100,000) against this substance could not be demonstrated. It caused temporarily the complete disappearance of precipitin, and though it may have appeared in the blood, in no instance was antigen and its precipitin simultaneously demonstrable. Apparently, the introduction of an antigen into a well immunized animal brings about a combination between antigen and antibody whereby the latter is temporarily removed from circulation. Its subsequent reappearance may either be due to the elimination of antigen or to the synthesis of new amounts of antibody.

These observations corroborate oft reported findings that with the progress of immunization the demonstration of antigen in the circulation becomes increasingly difficult. Wolfe and Dilks (1946) obtained immune sera from seven chickens, four immunized against goat and three against horse sera. Each chicken was bled 61 or 62 days after the last injection and immediately reinjected with the antigen previously used. Large decreases in antibody concentration were noted at two to five hours after the reinjection which was the earliest interval tried. For example, a chicken which had produced an antigoat serum precipitating at 1:102,400 dilution of antigen showed no precipitin in the serum obtained by bleeding four hours after the reinjection of the homologous antigen, and showed a titer of 200 after 22 hours. A

chicken which had produced antihorse serum precipitating with 1:102,400 dilution of antigen, showed no precipitin when bled five hours after the reinjection of homologous horse serum antigen, but the titer had risen to 409,600 when bled six days after the reinjection of antigen. The other chickens showed similar behavior in every respect.

Using quantitative chemical technique and horse serum and crystalline egg albumin as antigens, Culbertson (1935) in a similar study made the following observations:

After its injection into the circulation the horse serum as antigen persisted much longer in the blood of normal rabbits than of immune rabbits. In the normal animals, injected for the first time, complete removal of the antigen was never effected before the appearance of the precipitins in the blood.

All of the crystalline egg albumin introduced combines with circulating precipitin, which is immediately available for union with foreign protein. When the antigen is given in slight excess, a small residue of circulating precipitin generally remains in the blood. When large excess of antigen is given, antigen persists free in the circulation for about as long as an injection of similar size persists in the blood of a normal animal. The fixed tissue antibody is available in much less amount, and functions only when some of the antigen escapes union with the circulating precipitin and reaches the fixed tissues.

The above relationship between the circulating precipitin and homologous antigen is not altered by the injection of numerous non-specific substances into the circulation.

Hektoen and Welker (1935) also observed that the reduction or complete disappearance of antibody from the blood after introduction of the specific antigen, is sharply specific and that in the rabbit immunized against many antigens the injection of one of the antigens as the rule resulted in the disappearance from the blood of the precipitin for that antigen only. Lewis (1912) reported that diphtheria horse antitoxin, if injected into rabbits which had been previously injected with horse serum, was considerably less effective in neutralizing diphtheria toxin subsequently injected than in normal rabbits. Römer and Viereck (1914) reported that, in guinea pigs sensitized against horse serum, antitoxin injected into the blood disappeared more quickly than it did in normal animals. According to Hooker and Follensby (1931)



in human subjects markedly hypersensitive to horse serum, scarlet fever antitoxin when administered locally, loses its neutralizing effect for specific toxin more quickly than it does in non-sensitive persons.

The above cited observations show that in the presence of an insufficient amount of antibody production the antigen persists in the host and continues to exercise an effect until completely destroyed by the host enzymes. When an adequate amount of antibody is produced it may completely block the activity of an antigen. In an ordinary immunization experiment these conditions might correspond to the stage when the peak of antibody titer is reached, which then is followed by an abrupt decline of antibody production for the above stated reasons.

## B. THEORIES ON THE SITE OF ANTIBODY FORMATION

### 1. The Lymphocytic Theory

For over 40 years statements bearing on the lymphocytes as the site of antibody formation have been made by various investigators. Pfeiffer and Mark (1898) reported that anti-cholera antibodies appeared in the spleen, bone marrow, and lymph nodes before any increase could be detected in the blood. Anti-rabbit red cell hemolysins were reported to be present in serum, lymph from the thoracic duct, neck lymph, lymph from the limbs, salivary glands, etc. (Hughes and Carlson, 1908). Intravenous injection of antisera was followed by concentrations of antibodies in thoracic duct lymph greater than in cervical lymph (Becht and Luckhardt, 1916). Takahashi (1933) reported finding in the lymph of the rabbit anti-human red cell agglutinins flowing from lymph nodes, and believed that agglutinins were manufactured there. (For further detailed discussion see Drinker and Yoffey, 1941; Dougherty and White, 1947.) McMaster and Hudock (1935) summarizing numerous reasons, concluded that agglutinins are formed in the lymph nodes; and McMaster and Kidd (1937) observed the antiviral principle in the regional lymph nodes of rabbits which were injected with vaccinia in the ears.

Intensive studies on this question have since been carried out by Ehrich and Harris at the University of Pennsylvania, Dougherty, White and Chase at Yale University and Burnet and his co-workers in



Australia. Ehrlich and Harris (1942) injected typhoid bacilli or washed sheep erythrocytes subcutaneously in the hind legs of rabbits and determined the antibody titers in the afferent lymph, in the substance of the lymph node, in the efferent lymph, and in the serum. In a later study, Harris *et al.* (1945) separated the lymphocytes from the lymph plasma, prepared a cell extract and compared its content of antibody with that of the surrounding fluid. Ehrlich and Harris (1945), after a review of the observations pertaining to the reticulo-endothelial theory, summarized their results in the following manner. "When antigens were injected into the pad of the hind foot of the rabbit, antibodies first appeared two to four days after the injection in the lymph draining from the popliteal lymph node (the only node regional to the site of injection). They reached their highest titer after six days. In all experiments it was found that the antibody titer was higher in the efferent lymph; in some cases the concentration was 100 times that found in the lymph of afferent vessel. The production of antibody was preceded and accompanied by a rise in the output of lymphocytes in the efferent lymph which ranged from 15,000 to 20,000 per c.mm. to 60,000 to 80,000 per c.mm. or more. At the same time hyperplasia of the lymphatic tissue within the node occurred resulting in some experiments in a weight increase of the node from 0.2 g. to 1.0 g. or more. During antibody formation in the popliteal lymph node of rabbits, the lymphocytes in the efferent lymph vessels contain antibodies in a much higher concentration than the surrounding lymph. The ratio of titers amounted to from eight to 16 in many instances." Commenting on the lack of phagocytic activity of lymphocytes in relation to the antibody formation, and interpreting differently the results of Sabin (see page 72), they reason that the lymphocyte goes into action only after the raw material, i.e., bacteria or other formed antigens, have been properly prepared by the action of micro- or macrophages. In conclusion, they are of the opinion that the polymorphonuclear leucocytes and the macrophage as well as the lymphocyte may be instrumental in the antibody production, or through the cooperation of all these elements the immune bodies may be produced.

In a series of studies, Dougherty, Chase and White (1944), and White & Dougherty (1946), immunized mice against washed sheep erythrocytes. They found that the antibody titers in the extracts from three times washed lymphoid cells from immunized mice were ap-

proximately eight-fold higher than those in the corresponding sera on the basis of nitrogen contents. The absence of antibody titer in the final washings of the lymphoid cells showed that the antibody titer in the extracts was derived from the cells and not from adherent lymph. Salivary gland or muscle tissue, containing reticulum cells, macrophages, or fibroblasts, from the same immunized mice which had yielded antibody-containing lymphoid cells, showed no extractable agglutinins or hemolysins. Also lymphoid cell extracts from non-immunized mice were negative when tested for antibodies. Though these results seem to offer support to the theory that lymph nodes or lymphocytes might be the sites of antibody formation, these investigators stated that sites of antibody production and concentration other than lymph nodes may exist, e.g., bone marrow, spleen, liver, and other organs containing high proportions of reticulo-endothelial cells. These, however, were not examined. On the basis of the above results, though overwhelmingly favorable for the lymphatic system as the site for production of antibody, they were not inclined to conclude that lymphocytes necessarily are concerned with antibody formation.

Another study in this direction was reported by Kass (1945). Starting with a premise of questionable validity that if antibody is synthesized in lymphocytes, normal serum gamma-globulin should also be present, he prepared rabbit antisera to highly purified (electrophoretically 98 to 100 per cent pure) human gamma-globulin. These sera reacted specifically with extracts from human mesenteric lymph nodes obtained within one hour after death of a patient. Extracts of washed slices of human liver failed to react with the antiserum. This finding was interpreted to show the presence of gamma-globulin in lymphocytes and thereby the synthesis of antibody gamma-globulin within the same cell was assumed.

A few years previous to the publications of Ehrich and Harris, Harris, *et al.*, and Kass (cited above), Burnet, *et al.* (1938, 1941) reported the results of comparative studies pertaining to this question. Burnet and Lush (1938), immunizing mice with a virulent strain of influenza virus, found that antibody production occurred in the lymph node. Studying the spread of herpes virus and the formation of antibody in rabbits, they found that two out of five rabbits showed significant amounts of antibody in the lymph node at six and seven days after injection, while the results with another rabbit were weakly positive.

At this period there was no significant antibody in the serum, although by ten days considerable amounts were detectable. Similar results were obtained in experiments with dysentery agglutinin and phage. However, when staphylococcal toxoid was similarly studied, as the most convenient representative of these antigens, no clear evidence of antibody production in the local lymph node could be detected.

Since the primary antitoxic response was of such small extent, Burnet, *et al.* investigated the secondary response (specific anamnestic response) to the staphylococcal toxoid to detect antibody production in the lymph nodes. The toxoid was first injected intravenously; nineteen days later it was given subcutaneously in the right foot. A sharp rise in antitoxic titer of the serum had commenced on the 22nd day, and, believing that the secondary production of antitoxin was at its height, the rabbits were killed on the 24th day. The lymph nodes were removed, cleared of fat, and after weighing, they were emulsified (by grinding with quartz powder). After centrifuging the emulsion the filtered supernatant fluid was titrated for its antibody content. Expressing the values per gram of tissue they found that there was a considerable enlargement of the lymph node on the inoculated side, but there was only a barely significant difference in the antitoxic titers. Four days after the subcutaneous injections a low titer of antitoxin in the serum of rabbits was obtained, and there was no antitoxin in the left node, and only the smallest detectable trace in the right. In view of these and similar results they stated: "The general conclusion seems justified that antibody is produced by those phagocytic cells of the reticulo-endothelial system which ingest the antigen; which group is actually concerned in any particular instance will be determined by the site of infection of inoculation, and the subsequent spread of the antigen in the body."

a. **The Hormonal Control of Anamnestic Response and Lymphocytes.** It has frequently been observed that the amount of antibody in the circulatory system increases following the injection of a non-specific protein, or other non-antigenic substances. A reference has already been made (p. 33) to an observation that the administration of a non-specific substance, such as the plant alkaloid pilocarpin, stimulates the restoration of antibody production. This reaction has been known as the *anamnestic reaction*. This, as evidence for the increased production of antibody, has, however, not generally been accepted (Land-

steiner, 1945). In a series of studies, White and associates (White and Dougherty, 1944; Dougherty, White and Chase, 1944, 1947; Dougherty, Chase and White, 1945; Chase, White and Dougherty, 1946; White and Dougherty, 1946) recently reported that the increase in the antibody content of the blood stream subsequent to the administration of anamnestic agents is not a new antibody formation but the release of antibody concentrated in the lymphocytes. The injection of pituitary adrenotrophic hormone, or adrenal cortical extract caused changes in the lymphocyte tissue structure, decrease in the lymphocytes in the lymphatic tissue, increase in serum protein concentration and antibody titers. A chronic adrenotrophic hormone treatment diminished lymphoid tissues, produced a consistent lymphopenia and increased the serum protein levels. These changes did not occur in adrenalectomized mice injected with adrenotrophic hormone, though the lymphocytes of these same mice contained appreciable quantities of antibody. These investigators pointed out that this hormonal control of an important lymphocyte function integrates the role of lymphocyte and adrenal cortex in maintaining certain normal protective mechanisms of the organism.

Data show that one of the augmented serum protein fractions is that containing antibodies. These relationships are said to be normally under pituitary adrenotrophic hormone influence and may be altered by a variety of stimuli affecting pituitary-adrenal cortical activity, e.g., non-specific protein injections, hemorrhage, toxic chemicals, etc. Some of these have been demonstrated to produce pituitary-adrenal cortical activation resulting in lymphoid tissue dissolution, lymphopenia, and an increase in total serum proteins. Of these stimuli benzene and arsenite were reported to be very effective in producing the above mentioned alterations in normal mice but were totally ineffective in adrenalectomized or hypophysectomized mice. (See also Eisen *et al*, 1947.)

The anamnestic response occurring within three to nine hours in rabbits and mice following a single injection of either aqueous adrenal cortical extract, adrenal cortical steroid in oil, or adrenotrophic hormone showed both lymphocyte dissolution and globulin contribution to the serum. In this manner, it was shown that antibodies are an integral part of the lymphocytes, though not considered as evidence that lymphocytes are necessarily concerned with antibody formation.



**b. Findings Contrary to the Theory of the Hormonal Control of the Release and Fabrication of Antibody.** In contradiction to the findings discussed above, Eisen *et al.* (1947) reported that adrenal cortical activity is not essential for the fabrication or release of antibodies and gamma-globulin. These investigators found identical concentrations of serum antibodies and gamma-globulin in adrenalectomized rats repeatedly injected during immunization with adrenal cortical extract and similar animals not receiving this extract. Stoerk, *et al.* (1947) studied the effect of adrenal cortical hormones on the turnover of the serum proteins in adrenalectomized rats and likewise found that the administration of adrenal cortical lipo-extract did not exert any effect.

## 2. The Reticulo-Endothelial Theory

**a. The Theory of Sabin.** In a study to determine the true site and the mechanism of antibody formation, Sabin followed the course and the fate of R-salt-azo-benzidine-azo-egg albumin in the animal, and traced its distribution and disappearance in various body cells. The dye-protein in the form of purplish red-alum-precipitated particles was introduced into rabbits through intraperitoneal, intravenous, intradermal, and subcutaneous routes. After the intravenous injections the dye-protein was located in the Kupffer cells of the liver, in the macrophages of the milk spots of the omentum and in the corresponding cells of the peritoneal walls, as well as in the endothelium lining the lymphatic sinuses of the retrosternal nodes and in the free macrophages of sinuses and follicles of these nodes. After intradermal and subcutaneous injections it was in local macrophages and in the regional lymph nodes. The dye protein was found in the vacuoles of digestion of the phagocytic cell and altered first by the removal of the dye; thereafter the solid particles of protein disappeared and it was assumed to have been rendered soluble and passed into the cytoplasm. The vacuoles are considered the cellular organs of digestion, and the cytoplasm the zone of synthesis. Coincident with the time when the dye-protein is no longer visible within these cells, and when there are antibodies in the serum, there is observed a marked acceleration (over that of the normal rate) of the shedding of the surface films of the macrophages without damage to them. With the shedding



of parts of the surface films of these cells, both normal globulin and antibody globulin are carried out into the blood plasma. Thus it is stated that these mononuclear cells, functioning first as macrophages ("big eater"), render the antigen into suitable soluble form within the vacuole, and then as clasmotocytes ("shedding of exoplasm") incorporate it into the cytoplasm, and there in some way the increase in the synthesis of normal globulin and the modification of some of it into antibody globulin take place.

Coupling highly indiffusible blue dye T-1824, Evans blue, with various serum proteins and egg albumin, Kruse and McMaster (1949) prepared intensely blue dye-azo-proteins. A similar antigen consisting of extremely diffusible dye, echt-säure-blau, and of bovine  $\gamma$ -globulin was also prepared.

On intraperitoneal or intravenous injections, the azoproteins appeared in the Kupffer cells of the liver and sinus and reticular cells in lymph nodes, especially the great mesenteric node. These cells were particularly active in the removal of the blue antigens from the blood, but many other reticulo-endothelial cells were found to be active to a lesser degree. The storage of the antigenic material was observed in the cytoplasm only; it was not observed within nuclei, nor was it observed within the brain cells. Blue color was seen in the reticulo-endothelial cells of mice as long as  $3\frac{1}{2}$  months after injecting echt-säure-blau azoglobulin. The seizure of the blue azoprotein by reticulo-endothelial cells almost everywhere in the body was interpreted to indicate that antigenic stimuli to antibody formation can be brought to bear from practically all parts of the body upon those tissues or cells that are capable of antibody formation.

**b. Plasma Cells as Antibody Producers.** The hypothesis that plasma cells might be associated with process of immunization has been advanced by many authors on the basis of pathological-anatomical observations. In sternal bone-marrow punctures, a proliferation of plasma cells in lesions associated with hyperglobulinemia (infections, agranulocytosis, serum sickness and other morbid conditions) has been considered as affording clinical evidence in support of the hypothesis that plasma cells function as antibody producers.

*Plasma Cells (Plasmocyte, Phagocytes).* The plasma cell is described to be a cell rich in protoplasm, with eccentrically placed nucleus, relatively small, round or oval, with five to eight bands of chromatin

extending from the center like the spokes of a wheel; around the nucleus is a lighter zone, whilst the abundant protoplasm otherwise is dark, basophile. It has been claimed that there is a smooth transition between the lymphocytes and plasma cells. Others hold that plasma cells originate from cells belonging to the reticulo-endothelial system (Bing, 1940). Markoff (1937) stated that plasma cells of sternal bone marrow are identical with plasma-cellular reticulo-endothelial cells. According to him, reticulum cells of sternal marrow functionally can be divided into two groups: one, plasma-cell-like; the others, phagocytic and lymphoid forms.

Kolouch (1938) reported that there is an increase in the plasma cell content of the bone marrow running parallel with a rise in the antibody titer against *Streptococcus viridans*. According to Bing (1940) and Bing and Plum (1937) various diseases are accompanied by hyperglobulinemia, an increase of plasma cells and reticulo-endothelial cells in or outside of the bone marrow, which makes it probable that the formation of globulin takes place in these cells.

Bjørneboe and Gormsen (1941) immunized rabbits with various types of pneumococci killed with formalin. Intravenous injections were made every other day for a month, each injection containing 500 millions of pneumococci. Six of the immune sera contained 26 to 75 per cent more serum protein than the normal sera, or the globulin concentrations of immune rabbits were 6.1 to 12.9 mg./ml. of serum in comparison to 3.6 mg./ml. of normal serum. Histological examinations revealed a marked increase in plasma cells in spleen, in the capillaries of liver, plasma cell infiltration in the kidney and fatty tissues, slight plasma cell increase in the bone marrow. There was no increase in the reticulo-endothelial cells in these organs. They concluded that increase in globulin concentration of serum parallels the increase in plasma cells, or plasma cells are responsible for the synthesis of globulin.

In an extensive later study, Bjørneboe and Gormsen (1943), employing intensive intravenous immunization technique with several different antigens (polyvalent pneumococcal vaccine, polyvalent *Salmonella* vaccine and a mixture of proteins, etc.) simultaneously found the antibody concentration amounting to about 70 per cent of the total protein concentration of immune sera. Parallel with the rise of antibody globulin they observed an increase in plasma cells and young plasma cells with scanty protoplasm and without the characteristic

"spoke" arrangement of the chromatin of the nucleus, or merely a suggestion of this feature. Many of these cells correspond to the concept of "plasma cellular reticulum cells" which are taken to be the progenitors of plasma cells. Based on such careful analysis of various factors, they concluded that there is constant direct proportionality between the concentration of antibody formed and the degree of plasma cell proliferation in the organism which has been immunized.

Weighing various facts, one against the other, their reasonable explanation is that plasma cell proliferation essentially originates from elements of the reticulo-endothelial system, and as this system constitutes quite a considerable part of the spleen, the splenic weight is naturally increased by the proliferation in the reticulo-endothelial system. In other organs, for instance, the liver, the relative weight of the reticulo-endothelial system is too slight to influence noticeably the weight of the organ during the process of immunization.

Studying the cellular reaction in the rabbit spleen during the secondary response, after intravenous reinjections of antigens, especially living bacteria, Fagraeus (1948) observed a very strong plasma cellular reaction which was confined almost exclusively to the red pulp. The antigen (*Salmonella typhi*) was found in the periphery of the lymph follicles and in the red pulp, while but little antigen could be distinguished in the follicles. The great development of the plasma cells in the red pulp of the spleen after the injection of the antigen was thus preceded by the concentration of bacteria there. Successive stages of the above-mentioned intense plasma cellular reaction, associated with antibody formation, was accompanied by the finding of large reticulum cells or transitional cells followed 1 to 2 days later by immature plasma cells and, a few days later, an increase in the number of plasma cells. The plasma cells were usually not detected in the lymph follicles. In tissue culture studies, suggestive evidence of the production of antibody by spleen tissue was obtained. The antibody thus produced was from 20 to 200-fold in excess of the amount of antibody he could obtain in tissue extracts made from pieces of tissue from the parent spleen. Excised pieces of red pulp and lymph follicles, abundant, respectively, in plasma cells and lymphocytes, were separately studied for their capacities to form antibody. The antibody production by red pulp cultures was considerably greater than that by lymph follicle cultures. The production of antibody by the former

system was considered responsible for a reasonable portion of the total amount of circulating antibodies. Histological studies showed that tissues containing transitional cells were comparatively poor antibody-formers. The appearance of more mature cells was associated with an increase in the antibody content of the culture fluid. The decrease in the number of transitional cells was associated with an increase in antibody content of the serum, and the latter distinctly coincided with the number of immature cells showing growth. The transition of the immature plasma cells into mature cells was observed to lead to a decline in this capacity.

According to Caspersson (1947), all protein synthesis needs the presence of nucleic acids; quantitatively, the most important nucleic acids in the chromosome are of desoxyribose\* nature. The nucleus itself is a cell organelle organized especially for being the main center of the cell for the formation of proteins. In myeloma relatively large amounts of plasma cells appear in the blood. They show the typical picture of intense protein production (Thorell and Wising, 1944; Thorell and Wilton, 1945). Bing, Fagraeus and Thorell (1945) reported that a great amount of ribose nucleotides is found in the cytoplasm of the immature plasma cells. The nucleotide content together with the well developed nucleolar apparatus was considered to indicate an intensive production of protein in the cell. They consider the appearance of plasma cells as a sign that a conversion and an

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\*In a recent study Ehrich *et al.* (1949) observed that there is a parallelism between the increase in desoxyribonucleic acid and the increase in the weight of lymph nodes involving active cell multiplication. The peak of pentosenucleic acid increase was found to occur between the 4th and 6th day after vaccine injection when antibody formation was at its maximum. A histological study showed that on the 5th and 6th day mature plasma cells were the prevailing cells. Most of the pentosenucleic acid was contained in the plasma cells. A comparison of the rates of the proliferation of lymphocytes and plasma cells with the pentosenucleic acid and antibody formation showed that the plasma cells and not the lymphocytes are responsible for antibody formation.

A somewhat different point of view regarding the seat of the antibody synthesis is maintained by Harris and Harris (1949). After injecting antigenic and non-antigenic materials into the foot-pads of rabbits, the draining (popliteal) lymph nodes were daily studied histologically, chemically, and serologically. Non-antigenic materials showed no change. In response to antigenic materials the concentration of pentosenucleic acid rose to more than twice its normal value by the 2nd to 5th day followed by a decline. The peak of this change occurred at or slightly before the appearance of the maximal concentration of antibodies in the same node. The seat of these changes are believed to be the large, young lymphocytes, a view which differs from that which considers the plasma cells as the seats of antibody synthesis.



intensification of the protein production has occurred in reticulo-endothelial cells, incited by the great antigen doses injected. From a functional point of view, this development culminates in the formation of the immature plasma cells. Maturing of the latter into plasma cells marks the transition to a less active state, and, therefore, the mature plasma cell represents the final link in a chain of development, a cell which has already passed the stage of its great functional intensity. Fagraeus (1948) concluded that "*reticulo-endothelial elements under the conditions described, produce antibodies, thereby developing into a type of cell with the morphological characteristics of the plasma cell.*" She found no evidence which would directly favor the participation of the lymphocytes in the formation of antibodies.\*

### C. THEORIES ON THE MECHANISM OF ANTIBODY FORMATION

In Part I, experimental data were cited to indicate that the rôle of antigen in the production of antibody might be one of catalysis. It was further stated that naturally occurring iso-antibodies, agglutinins against bacteria, and red blood cells, precipitins against animal and plant proteins and toxins appeared to be serum globulins. These would emphasize not only the fact that the normal serum and antibody globulins are closely related but also the fact that the site of their formation might be identical.

At the present time, we are handicapped by the lack of precise

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\*Fagraeus (1947) found that the thymus, where the chief production of lymphocytes takes place (Andreasen and Ottesen, 1945), has an insignificant antigen-phagocytizing capacity, and lacks entirely the capacity to form antibodies *in vitro*. Harris, Rhoads and Stokes (1948) also studied the function of thymus in relation to the formation or retention of antibodies. They likewise found that the thymus did not play a demonstrable role in the formation of antibodies by the young rabbit, or in the retention of antibodies derived by the fetus from the maternal circulation. The formation of antibodies by spleen was also comparatively studied. They found slight evidence of formation of antibodies in the spleen following *subcutaneous* injections, but high antibody-titers were demonstrated in the spleen extracts following *intravenous* injection of antigens. This difference was explained by assuming that "the subcutaneously injected rabbits may well have formed the bulk of their antibody to the *Shigella* in the lymph nodes draining the site of injection, the spleen being stimulated only by the fraction of soluble antigen-specific material which reached it via the blood stream. In the intravenously injected animals, on the other hand, the spleen could have received a major portion of the injected antigen and would thus have played a relatively greater role in the total antibody response of the animal."



knowledge as to the finer structure of proteins to formulate a comprehensive mechanism of antibody formation for correlating its chemical and physical properties with that of the specificity of serological reactions. In this connection it might not be irrelevant to review some of the properties of proteins pertinent to our question.

## 1. An Introductory Comment on the Antigenic Specificity of Proteins

We know that the proteins differ in a general way in their physico-chemical properties—solubility, iso-electric point, electric charge, molecular weight and shape, etc. We do not know very much about the spatial configuration, but we know that one protein might differ from others in the arrangement and content of amino acids. A protein might be simple or conjugated with nucleic acid, heme, coenzyme, carbohydrate, lipid, etc., but none of this information has as yet clarified the subject. It has further been known that all proteins are constructed of a large number of  $\alpha$ -amino acids which are combined through peptide bonds, thus forming a long chain containing many CO-NH groups or peptide bonds. The proteins contain (in the absence of  $\epsilon$ -amino acid) practically no free amino or  $\alpha$ -carboxyl groups. Consisting solely of l-amino acids with the exception of glycine, which has no asymmetric carbon atom and thus no optical activity, the proteins have uniform optical configuration. Specificity of proteases is singularly limited to the hydrolysis or synthesis of molecules of l-configuration. These enzymes have no effect on the d-amino acids. For example, glycine (glycocoll or  $\alpha$ -amino-acetic acid,  $\text{NH}_2\text{CH}_2\text{COOH}$ ) and alanine ( $\alpha$ -amino-propionic acid,  $\text{CH}_3\text{CHNH}_2\text{COOH}$ ) can form four different dipeptides: (1) glycylglycine, (2) alanylalanine, (3) glycylalanine, (4) alanylglycine. It is significant that peptides 3 and 4, although giving the same amino acids on hydrolysis, are not the same. This gives some clue to the possibilities of combinations of the amino acids in proteins. It can be seen that when the number of amino acids available for such head-tail amide formation is increased, the number of combinations due to the order in which they are linked increases very rapidly. Emil Fischer (1923) starting with glycylglycine synthesized an octadecapeptide with a molecular weight of 1213. He calculated that out of 30 amino acids, 18 of which

are dissimilar,  $1.28 \times 10^{27}$ , and out of 20 naturally known amino acids  $2.4 \times 10^{18}$  protein isomers could be formed. It must be understood that such a polypeptide, as well as any protein, exists, or can exist in numerous tautomeric forms, each of which will have properties different from the other.

In proteins the amino acids are bound together by peptide bonds through  $\alpha$ -amino and  $\alpha$ -carboxyl groups and the rest of the amino acid is essentially free. The non-peptide forming parts of the amino acids constitute side groupings in a protein molecule. The amino acids are listed below (Table I); according to their side groupings they contribute to the structure of the protein molecule. As will be seen, they are acidic, basic, polar and hydrophobic. These characteristics are related to free carboxyl, amino, amide, guanidine, phenolic, aliphatic hydroxyl, indole imino, imidazole NH, methyl sulfide, disulfide groups, and phenyl and aliphatic hydrophobic hydrocarbon radicals.

Studies with enzymes, hormones, viruses (simple), and antigens deal with specific chemical reactions with certain of these side groupings in relation to their reversible and irreversible inactivation. Despite extensive investigation the nature of enzymatic and antigenic specificity of the proteins remains the challenging unsolved problem. It is true, for example, that the oxidation of SH groups, or the reduction of -S-S- groups destroys the activity of certain proteins, but this fact alone does not explain the specific activity of various enzymes to which the same reactive SH or -S-S- groups are attached. There is no doubt that these specificities are governed by the whole protein molecule or parts thereof larger than the SH or similar groups.

The integrity of every constituent part of the protein molecule functioning as virus, enzyme, hormone or antigen is essential for full biological activity. A scission in the molecule usually causes complete loss of activity. In this respect, the ability of an antigen to initiate the production of a specific antibody is comparable to those of enzymes and hormones. Splitting of a protein of minimal molecular unit size causes loss of the ability to stimulate antibody formation. This property of antigen being catalytic in character differs from its ability to combine with homologous antibody. In the latter case the reaction is stoichiometrical, is independent of molecular size and, therefore, of the integrity of the whole antigenic molecule. As is well known, the split products, as haptens, enter into stoichiometrical combination with

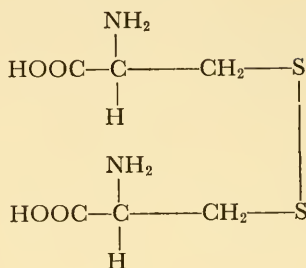
TABLE I

AMINO ACIDS WHICH YIELD BASIC, ACIDIC, POLAR AND HYDROPHOBIC SIDE GROUPS IN THE PROTEIN MOLECULES

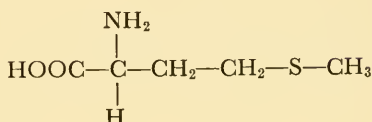
Name of amino acid	Structural formula
Amino Acids With Basic Side Groups	
1(+)-Lysine ( $\alpha$ - $\epsilon$ -diamino-caproic acid)	$\begin{array}{c} \text{NH}_2 \\   \\ \text{HOOC}-\text{C}-(\text{CH}_2)_4-\text{NH}_2 \\   \\ \text{H} \end{array}$
1(+)-Arginine ( $\delta$ -guanido- $\alpha$ -amino-valeric acid)	$\begin{array}{c} \text{NH}_2 \qquad \qquad \text{H} \\   \qquad \qquad \quad   \\ \text{HOOC}-\text{C}-(\text{CH}_2)_3-\text{N}-\text{C}=\text{NH} \\   \qquad \qquad \qquad   \\ \text{H} \qquad \qquad \quad \text{NH}_2 \end{array}$
1(-)-Histidine ( $\beta$ -imidazol- $\alpha$ -amino-propionic acid)	$\begin{array}{c} \text{NH}_2 \\   \\ \text{HOOC}-\text{C}-\text{CH}_2-\text{C}=\text{CH} \\   \qquad \qquad \quad   \quad   \\ \text{H} \qquad \qquad \quad \text{N} \quad \text{NH} \\ \qquad \qquad \quad // \quad \backslash \\ \qquad \qquad \quad \text{CH} \end{array}$
Amino Acids With Acidic Side Groups	
1(+)-Glutamic acid ( $\alpha$ -amino-glutaric acid)	$\begin{array}{c} \text{NH}_2 \\   \\ \text{HOOC}-\text{C}-\text{CH}_2-\text{CH}_2-\text{COOH} \\   \\ \text{H} \end{array}$
1(-)-Aspartic acid ( $\alpha$ -amino-succinic acid)	$\begin{array}{c} \text{NH}_2 \\   \\ \text{HOOC}-\text{C}-\text{CH}_2-\text{COOH} \\   \\ \text{H} \end{array}$
1(-)-Tyrosine ( $\beta$ -p-hydroxy-phenyl- $\alpha$ -amino-propionic acid)	$\begin{array}{c} \text{NH}_2 \qquad \text{H} \\   \qquad \quad   \\ \text{HOOC}-\text{C}-\text{C}-\text{C}_6\text{H}_4-\text{OH} \\   \qquad \quad   \\ \text{H} \qquad \quad \text{H} \end{array}$
1(-)-Cysteine ( $\beta$ -thiol- $\alpha$ -amino-propionic acid)	$\begin{array}{c} \text{NH}_2 \\   \\ \text{HOOC}-\text{C}-\text{CH}_2-\text{S}-\text{H} \\   \\ \text{H} \end{array}$

### Amino Acids With Polar Side Groups

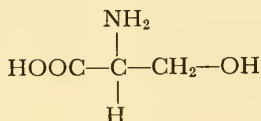
1(—)Cystine [di-( $\beta$ -thiol- $\alpha$ -amino-propionic acid)]



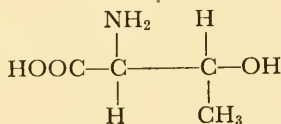
1(–)-Methionine ( $\gamma$ -methylthio- $\alpha$ -amino-n-butyric acid)



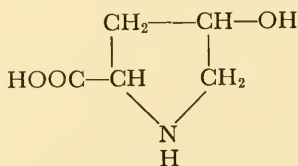
1(—)-Serine ( $\beta$ -hydroxy- $\alpha$ -amino-propionic acid)



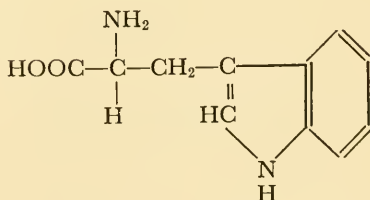
1(—)-Threonine ( $\alpha$ -amino- $\beta$ -hydroxy-  
n-butyric acid)



1(-)-Hydroxy-proline (4-hydroxy-pyrrolidine-2-carboxylic acid)

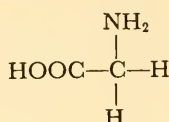
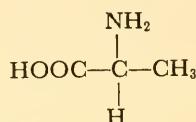
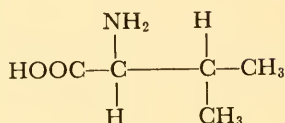
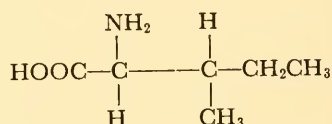
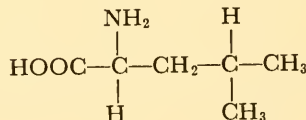
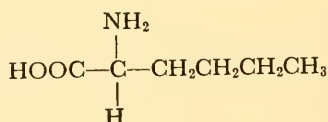
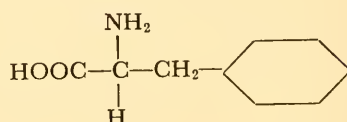


1(-)-Tryptophane ( $\beta$ -3-indole- $\alpha$ -amino-propionic acid)

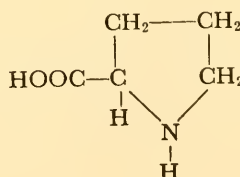


## Amino Acids With Hydrophobic Side Groups

Glycine (glycocol, amino-acetic acid)

1(+)-Alanine ( $\alpha$ -amino-propionic acid)1(+)-Valine ( $\alpha$ -amino-isovaleric acid)1(+)-Isoleucine ( $\beta$ -methyl- $\alpha$ -amino-valeric acid)1(-)-Leucine ( $\alpha$ -amino-isocaproic acid)Norleucine ( $\alpha$ -amino-caproic acid)1(-)-Phenylalanine ( $\beta$ -phenyl- $\alpha$ -amino-propionic acid)

1(-)-Proline (pyrrolidine-2-carboxylic acid)





antibody. The specific combining capacities of antigens reside, therefore, in the whole as well as in its component small molecular weight non-antigenic units. These units carry specific configuration and chemically reactive groups. On the basis of these established facts one may conclude that while the specific combining capacities of antigens, or their component units, are dependent on the amino acid composition and order of their linkage yielding specific configuration to each protein molecule, the antibody producing catalytic activity depends on the integrity of the whole molecule.

The interdependence between catalytic activity of antigen and its minimum irreducible molecular unit size is a common and basic property of enzymes, hormones and viruses also. No doubt, the specificities of the latter three are similarly dependent on the amino acid composition and order of their linkage yielding specific configuration to each species of protein molecule.

These facts may perhaps more fully be appreciated if we consider the fact that the specific activities of the same heme group in various closely related enzymes, cytochromes, cytochrome oxidase, cytochrome peroxidase and catalase, are directed by specific protein carriers. Similarly, there exist distinct differences in antigenic specificity of closely related classes of proteins. One can cite as an example differences in this respect of a class of  $\alpha$ -,  $\beta$ - and  $\gamma$ -globulins from the same species of animal. Kendall (1937, 1938) showed that these globulins of human serum are antigenically specific. The antibodies against these globulins were found to be specific for each of these globulins; that is, the  $\alpha$ -globulin antiserum reacted with the  $\alpha$ -globulin to give a precipitate, but did not react with  $\beta$ - or  $\gamma$ -globulin, with albumin, or with any other component of human serum. Thus, this highly refined immunological differentiation of the closely related globulins of human serum, which differ from each other in amino acid composition (Table II), electrical mobility, molecular weight, viscosity and isoelectric point, makes the correlation between the structural pattern and the biological specificity of the proteins reasonably plausible.

TABLE II  
AMINO ACID COMPOSITION OF HUMAN PLASMA PROTEINS  
*g per 100 g. Protein*

Constituent	Albumin	$\gamma$	$\beta$	$\alpha$	Fibrinogen
Total N	15.95	16.03	15.24	...	16.9
Total S	1.96	1.02	1.32	...	1.26
Free $\alpha$ -amino N	0.18	0.11	...	...	...
Amide N	0.88	1.11	...	...	...
Glycine	1.6	4.2	5.6	3.1	5.6
Alanine	...	...	...	...	...
Valine	7.7	9.7	7.0	5.2	4.4
Leucine	11.0	9.3	7.9	14.2	7.1
Isoleucine	1.7	2.7	5.0	1.7	4.8
Proline	5.1	8.1	7.1	4.7	5.7
Phenylalanine	7.8	4.6	4.7	4.6	4.2
Cysteine	0.7	0.7	{ 3.5 }	{ 1.5 }	0.4
Half-Cystine	5.6	2.4			2.3
Methionine	1.3	1.1	1.7	1.4	2.5
Tryptophan	(0.2)	2.9	2.0	1.9	3.3
Arginine	6.2	4.8	6.8	7.7	7.9
Histidine	3.5	2.5	2.8	2.8	2.8
Lysine	12.3	8.1	6.6	8.9	8.3
Aspartic Acid	10.4	8.8	9.8	9.0	13.6
Glutamic Acid	17.4	11.8	14.5	21.6	14.3
Serine	3.7	11.4	7.1	5.0	9.2
Threonine	5.0	8.4	6.1	4.9	6.6
Tyrosine	4.7	6.8	6.0	4.5	5.8
Totals	105.9	108.3	104.2	102.7	108.8

John T. Edsall (1947): *Advances in Protein Chemistry*, Volume III. Edited by M. L. Anson and John T. Edsall. New York, Academic Press, 1947. p. 464.

## 2. The Role of Determinant Groups on the Antigenic Specificity of Conjugated Proteins

Although all attempts with natural proteins have failed to obtain an answer to why one protein is antigenically different from the other,

studies in another direction by Landsteiner and his followers have shed considerable light on the rôle of the "determinant groups" in antigens on the specificity of antibodies. There is though no evidence that the natural simple proteins contain characteristic "determinant groups" to account for the specificity of homologous antibodies; however such groups introduced into the artificial proteins have been shown, by numerous examples, to exercise the power of determining the specificity of antibodies against them.

Of the considerable number of such examples, which will be taken up in a later chapter on the similarity of the specificities of enzymes and antigens, we will consider at this point only two of the most outstanding examples. It is to be noted that these examples have more or less served as a basis for the theories on the mechanism of antibody formation. Landsteiner and van der Scheer (1928, 1929) demonstrated that d- and l-*para*-amino-tartranilic acids coupled with proteins produce antibodies specifically reactive. Avery and Goebel (1929) similarly showed that p-amino-phenol- $\beta$ -glucoside and p-amino-phenol- $\beta$ -galactoside coupled with a protein produce respectively specific antibodies. In these examples we have the differences between the "determinant groups" reduced to the simplest structural forms known to chemistry. In tartranilic acid antigens the apparent difference is one of optical or space isomerism. In p-amino-phenol- $\beta$ -hexoses likewise the difference is one of space isomerism. These differences are associated also with other chemical properties, such as differences in the reactivity to specific enzymes, solubilities, and also physical and chemical differences of their respective derivatives. How are we then to interpret the specificity of antibodies produced against them? Does it signify that antibody against d-antigen possesses l-configuration and vice versa? Or are we to assume that antibody against one is the space isomer of the antibody against the other? Or else must we assume that chemical and physical differences resulting from or associated with the difference of optical or space isomerism are responsible for the differences in the specificities of antibodies against each and every one of them? A satisfactory answer to these questions has an important bearing on the formulation of the concepts of the structure of antibodies in relation to the nature and the position of the serologically reactive specific groups. At present our knowledge regarding these questions is not of sufficient scope to formulate the answer satisfactorily;

we must therefore confine our ideas for the time being to the realm of possibilities.

With these limitations in mind, the theories advanced concerning the *mechanism of antibody formation* are discussed below.

### 3. Mechanism of Antibody Formation

(a) Breinl and Haurowitz (1930), (b) Mudd (1932), (c) Alexander (1931), (d) Pauling (1940) and others have offered hypotheses on the possible mechanism of antibody formation.

**a. The Theory of Breinl and Haurowitz.** These authors assume that the cells which synthesize serum globulin possess species specific surfaces containing certain groups with residual valences which attract or repulse amino acids participating in the formation of globulin. An antigen combining with these surfaces produces new groups of different residual valences. The orientation of amino acids in the formation of antibody globulin are thus governed by surface valences of the new cell-antigen complex. Possible evidence concerning the presence of surface valences are cited from the observations of Landsteiner that only those antigens containing groups with weak residual valences, such as  $-\text{COOH}$ ,  $-\text{NH}_2$ ,  $-\text{HSO}_3$ , etc. show antibody determining property whereas aliphatic (paraffin) chains, or plain aromatic rings, which lack such residual valences, are devoid of antibody forming property.

**b. The Theory of Mudd.** Mudd bases his concept of the mechanism of antibody formation on the specific relationship between antigen and antibody. The striking instances cited by him are the d- and l-tartranilic acid azo-proteins of Landsteiner and van der Scheer (1929) and the p-amino-phenol- $\beta$ -glucoside and p-amino-phenol- $\beta$ -galactoside azoproteins of Avery and Goebel (1929). Injected into rabbits they give rise to antibodies which combine electively with the particular stereoisomer injected. Synthesis by the linking of amino acids to the peptide is supposed to occur in an orienting environment, namely the antigen-protoplasm interface. The chemical groupings linked to the molecule undergoing synthesis at the antigen surface are believed to be adapted spatially and in their chemical affinities to the antigen surface at the region in which linkage occurs. The synthesized antibody molecule should therefore possess to some degree a stereochemical

correspondence with the antigen for the reason that each structural unit has been selected and oriented to fit the local configuration and affinities of the antigen surface. Thus, an antibody "specific" for the antigen, i.e., possessing specific stereochemical correspondence with it, is supposed to have been formed.

c. **The Theory of Alexander.** According to Hektoen the minimum sensitizing dose of egg albumin is approximately 0.000,05 mg. It has also been frequently shown that if an animal be bled, the temporary drop in the titer of antibodies in the remaining blood is retrieved and even surpassed. In view of this great disproportionality between the antigen used and the antibody produced, Alexander points to a similarity between these facts and the rôle of enzymes or catalysts. He believes that the antigen forms within the (antibody forming) cells themselves, new specific catalysts which are able to direct the formation of antibodies. In this connection three possibilities are considered: (a) modification of a gene, (b) modification of a non-genic catalyst, and (c) fixation of the antigen particle by a non-catalyst cytoplasmic particle in such a manner that the combination functions as a specific catalyst. Variations in the duration of immunity are assumed to correspond to variations in the persistence of the antigen-catalyst complex, while inability to establish immunity would indicate the non-formation of such a complex.

As will be discussed on pages 104-8 of this treatise we do not subscribe to the idea of Alexander that an antigen produces a modification of a gene. Our view concerning this question basically differs from that of Alexander.

d. **The Theory of Pauling.** In disagreement with Breinl and Haurowitz, and Mudd, Pauling does not believe that the effect of an antigen in determining the structure of an antibody molecule is the ordering of the amino-acid residues in the polypeptide chains in a way different from that in the normal globulin. Hypothetically he assumes that *all antibody molecules contain the same polypeptide chains as normal globulin, and differ from normal globulin only in the configuration of the chain; that is, in the way that the chain is coiled in the molecule.* It is further stated that "the number of configurations accessible to the polypeptide chain is so great as to provide an explanation for the ability of an animal to form antibodies with considerable specificity for an apparently unlimited number of different antigens, without the



necessity of invoking also a variation in the amino-acid composition or amino-acid order."

A globulin molecule is pictured as consisting of a single polypeptide chain, containing several hundred amino-acid residues. The arrangement of the amino-acid residues in the central part is much more stable than any other, whereas the two end parts of the chain are of such a nature that there exist for them many configurations with nearly the same energy. "The atoms and groups which form the surface of the antigen will attract certain complementary parts of the globulin chain (a negatively-charged group, for example, attracting a positively-charged group), and repel other parts. As a result of these interactions the configurations of the chain ends which are stable in the presence of the antigen will be such that there is attraction between the coiled globulin chain ends and the antigen, due to their complementarity in structure. The configuration assumed by the chain end may be any one of a large number, depending upon which part of the surface of the antigen happens to exert its influence on the chain end and how large a region of the surface happens to be covered by it." The middle part of the antibody molecule thus produced would be like that of a normal globulin molecule. Hence antibodies should have antigenic activity, with essentially complete cross reactions with normal globulin. The two ends, on the other hand, would have configurations more or less complementary to parts of the surface of the antigen. According to this picture the active end regions of the antibody molecule would not have effective antigenic power.\* This is explained by assuming that the configurations of the end regions would be different from molecule to molecule, and that an antibody complementary to one antibody end would as a rule not combine with another.

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\*The concept of Pauling appears to be in contradiction to the results provided by the studies of Northrop (1942). He reported that diphtheria antitoxin (mol. wt. 184,000) on digestion with proteolytic enzymes was obtainable in crystalline form. This substance was 90 per cent precipitable with diphtheria toxin and had a molecular weight of 90,000. The immune sera prepared against the crystalline substance were active against antitoxin but inactive against the normal serum proteins.

The fact that the crystalline substance represents one-half of the original antitoxin molecule, fails to support the hypothesis of Pauling that the antibody molecule is made up of a stable midpiece carrying at each end polypeptide chains susceptible to configurational changes. In view of Northrop's results it would seem difficult to label certain parts of the antibody molecule as corresponding to mid or end pieces.

#### 4. The Concept of Catalysis as Implied by the Above Theories on the Mechanism of Antibody Formation

The above discussed theories on the mechanism of antibody formation imply concepts which support the view presented in this treatise that the antigens exercise the rôle of catalysts in the formation of specific antibodies. The following statements concerning this question are taken from the above discussed theories: "cells which synthesize serum globulin possess species specific surfaces," and "antigens combining with these surfaces produce . . . new antigen-cell complexes directing the synthesis of antibody globulins" (Breinl and Haurowitz); "antigen-protoplasm interface" serving as an orienting environment in the synthesis of antibodies (Mudd); "the antigen molecule, after its desertion by the newly-formed antibody molecule, may serve as the pattern for another" (Pauling), which is a function of all catalysts; "Macrophages render the antigen into suitable soluble form within the vacuole and then as clasmatocytes incorporate it into the cytoplasm, there in some way increasing the synthesis of normal globulin and the modification of it into antibody globulin . . ." (Sabin); "antigen forms within the (antibody forming) cells themselves, new specific catalysts which are able to direct the formation of antibodies" (Alexander), are concepts which support the thesis that the mechanism of immunization is similar to the mechanism of surface catalysis in heterogeneous systems. The similarity between the experimental facts serving as a basis for the above views, and those to be encountered quite extensively in chemical processes, where catalysis plays a determinant rôle, is quite clear.

We know that normal and immune serum globulins are synthesized probably within reticulo-endothelial cells (Sabin). To account for the specific reactivity of the antibody globulin it is necessary to assume the formation of a new cell-antigen catalytic surface. Evidently the cell as such is acting as an anchor or a catalytic support for antigen to exercise its directive influence in modifying the synthesis of normal globulin into antibody globulin. These modifications of surfaces within cells may be pictured as analogous to the mechanism of heterogeneous catalytic surfaces. They remind us of the well-known "mixed" or "supported" catalysts, which represent mixtures of two or more sub-

stances, capable of producing a greater specific catalytic effect than can be accounted for if they were to act independently.

## 5. The Theory of Burnet, *et al.* of Antibody Formation and Adaptive Enzyme Process

A valuable contribution to immunology is the monograph, *Production of Antibodies*, by Burnet, *et al.* (1941; Burnet and Fenner, 1950). Among other topics, the chapter on the "*Theoretical Aspects of Antibody Production*" concerns us here most. Because of the war conditions this publication was unknown to us, hence no reference to this theory will be found in the 1st edition of *Immuno-catalysis* which went to press in the summer of 1943.

Burnet, *et al.* advance a theory of antibody production which, in essence, is that antibodies are produced in a manner analogous to the "production" of so-called "adaptive enzymes." The theory postulates that *antigen specifically modifies the proteinase producing a new enzyme with the ability to synthesize an antibody specifically reactive with the antigen.* The basic tenets of this concept, as will be discussed below, conflict with the experimental basis of the existing theories dealing with the interrelationship of genes and enzymes, the changes a cell may undergo as a result of mutations, and the chemical activities of the cells as the seats of enzyme action, etc. Before we undertake an analysis of these factors it is necessary that the salient points which Burnet, *et al.* offer in support of their theory be presented.

### a. The Premises of the Theory of Burnet, *et al.*

- (1) "*Antibody is composed of globulin molecules which are produced by and liberated from those cells of the reticulo-endothelial system which ingest the antigenic molecules or particles.* Particulate antigens introduced into the tissues are largely dealt with by cells of the lymph nodes, while, if they (the antigens) reach the blood stream, cells of the spleen, liver and bone marrow are chiefly concerned. There is still some doubt as to where bacterial antitoxins are produced. From Buttle's experiments (1934) the cells of the bone marrow may represent the main source of antitoxin.
- (2) "*A second or subsequent contact with the same antigen*

*provokes a more active production of antibody.* This is seen more clearly with toxoids than with particulate antigens, but when looked for can be observed with all types of antigens. The latent period is shortened, the antibody titer rises more rapidly and to a higher titer, and the rate of subsequent fall is slower.

- (3) *"Antibody in the circulation is being constantly removed at a rate which is approximately proportional to its concentration.* This is based largely on the data from passive immunity experiments with sera of the same species, but adequate reasons have been given for assuming that it holds also for actively produced antibody.
- (4) *"Antibody production following an antigenic stimulus rises to a peak and then diminishes, but continues at a diminishing rate often for long periods.* The classical example is the persistence of demonstrable yellow fever antibody more than fifty years after the last contact with the virus.
- (5) *"Antibody production can continue long after the antigen responsible has disappeared from the body.* This conclusion is perhaps debatable, but reasons have been given earlier for adopting it. The alternative, to suppose that the cell retains the antigen unmodified but undetectable by any experimental method, is against all biological analogies, creates grave difficulties in interpreting qualitative changes in antibody with repeated immunization, and could only be adopted if no other interpretation could account for the facts.
- (6) *"Antibody production is a function not only of the cell originally stimulated, but of its descendants.* The cells of the reticulo-endothelial system are well known to vary enormously in number and in response to physiological and pathological stimuli. The inoculation of antigenic and non-antigenic foreign material is one of the most potent methods of inducing their proliferation. Although direct proof is impossible, it is a reasonable assumption from this lability in numbers that the life of any of these cells is a short one, and that when antibody production goes on for months or years other cells than those initially stimulated must be responsible." Rejecting the persistence of thorium dioxide in the



reticulo-endothelial cells of liver and spleen for years as evidence for the persistence of the same individual cells, Burnet concluded: "we can see no escape from the conclusion that the antibody-producing mechanism can be transmitted to descendant cells by some hereditary process.

- (7) *"The type of antibody produced varies (a) according to the species used, (b) with the age of the animal, and (c) according to the nature and frequency of the antigenic stimuli. The change in character of the antibody following repeated re-inoculation is the difference of most theoretical importance. It would indicate that an antibody-producing mechanism once established can be further modified by new antigenic contact."*

Of the criteria offered by Burnet, *et al.* in support of their theory, criteria one to four can be explained by the observations considered in the preceding discussions in relation to the factors controlling the immune response to an antigen, and those related to the antigen-antibody balance during the process of immunization. The most challenging of these criteria are those condensed in paragraphs five and six. It is here assumed that antibody production continues long after the antigen has disappeared from the body. It is, therefore, concluded that antibody production is a characteristic which is acquired not only by the cell proteinases but also transmitted to the descendant cell proteinases. In other words, it is an acquired characteristic inheritable by generations of cells during the lifetime of the host. In support of these assertions these authors cite, in particular, the lifetime persistence of immunity against measles and yellow fever virus. In the case of yellow fever, based on an observation by Sawyer, they stated that "there is direct evidence that a single infection may induce the formation of antibody which can be detected in the serum 75 years later."

Landsteiner (1945), in reference to the nature of long-lasting immunity, and certain claims that antigens leave impressions upon the antibody-producing cells which last after the disappearance of the antigen, made the following statement: "The fact that immunity can last for many years would be a decisive proof, but in the most striking case of virus infections (smallpox, measles, yellow fever), the permanence of active virus cannot be excluded." According to Rous (1946),



the viruses may lie latent permanently in plants and animals yet cause no discernible harm, e.g., the virus of King Edward potato plants, which cause them no perceptible trouble, though capable of killing plants of other varieties, or the virus from human "fever blisters" which causes encephalitis in an inoculated rabbit. The "latent" viruses stimulate in the animal host the formation of specific protective antibodies.

According to Dixon (1945), a lasting immunity to viruses demonstrable by complement fixation, agglutination, precipitation and neutralization following one attack in an animal, may depend on the kind of tissue infected and is probably due to a long-term sojourn or persistence throughout the life of the host. Lasting immunity may not be obtained to influenza or to the common cold virus because the superficial cells lining the respiratory tract are being thrown off at intervals to be replaced by deeper cells and thus do not provide a permanent abode for these viruses. It may further be stated that all studies so far carried out, and intended to determine the relation of the duration of antigen to antibody production in a host, show that the disappearance of antigens (some tagged with radioactive atoms) from host tissue results in the cessation of antibody production (Ehrich, *et al.* 1945; Herdegen, *et al.* 1947; Libby, 1947).

According to Taliaferro (1932, 1938) when a trypanocidal crisis is permanently effective, it is because the few parasites which escape destruction cannot produce a relapse because they are prevented from reproducing by anti-reproduction (ablastin) antibody. According to Packchianian (1934) when the animal has recovered from reinfections, it is quite possible that all the trypanosomes (*Trypanosoma brucei*) have been destroyed, at least in the blood. If a few latent ones do exist, they are so attenuated that they cannot produce any detectable infection in subinoculated test animals. Those reinoculated forest deer mice are considered relatively immune to further reinoculation with *Tr. brucei*. Experimental data indicated that even in certain individuals of forest deer mouse (*P. L. noveboracensis*), prolonged and indefinite latency was associated with a few surviving trypanosomes. Thus, 100 days after the initial inoculation, the animal was etherized and almost its entire heart blood, 0.5 ml., was injected into a rat. The latter animal was observed for five months and failed to show symptoms of nagana. The heart, lungs, liver, spleen, kidneys, brain, muscles, and part of the bone marrow and glands of the same mouse were ground into pulp in

an equal volume of tyrode solution and introduced into the peritoneal cavity of rabbit by means of regular surgical technic. This rabbit later developed symptoms of nagana and trypanosomes were demonstrated in its blood. Two drops of ear blood from this animal were inoculated into a rat which contracted the disease and died after 8 days. Fifty-three days after the original inoculation, this rabbit was found dead. This particular experiment shows that at least a few trypanosomes were present in the tissues or organs of the forest deer mouse, an animal which had been relatively immune on the basis of negative microscopic examination of its blood, as well as on the basis of the noninfectivity of its blood to rats.

In another set of studies Packchanian and Tom (1943) reported the presence of agglutinins for *Leptospira icterohaemorrhagiae* in the circulating blood of patients for periods from one year to at least 20 years and seven months after recovery from Weil's disease. It is interesting that in all cases during the periods indicated there has been a decline in the agglutinin titer. However, the sera were never free from agglutinins of significant titer. Following up these findings Packchanian and Sonnier (1948) found that tests for agglutinins in the serum samples of 18 rats (*R. norvegicus*) were negative and no *Leptospira* were found in the kidneys of these rats. However, the serum samples of a wild rat, whose kidneys were positive for motile *Leptospira*, gave agglutination reaction with Type I *Leptospira icterohaemorrhagiae* in dilutions up to 1:30,000 and the serum sample from an infected mouse, whose kidneys also were positive for *Leptospira*, gave an agglutination reaction in dilutions up to 1:10,000.

These investigators have shown that less virulent strains of *Leptospira* may fail to produce death in guinea pigs, but may produce antibodies, such as agglutinins and lysins as a result of subclinical infection.

Burnet, *et al.* interpret the above claimed long-lasting immunity in the following manner. The fractions of the serum globulin which are concerned in immunological reactions are synthesized by cellular proteinase units. These liberate partial replicas of themselves. These proteinases, by virtue of their enzymatic function, come into contact with any foreign antigens taken into the cell, are lastingly modified by this contact. The modification of the proteinase unit which is produced by contact with the antigen is not to be regarded as resulting from a synthesis of a new unit in spatial contact with antigen, but rather as a

process analogous to the production of adaptive enzymes by bacteria. The stress is laid on the assumption that contact with a sugar molecule not normally fermented can impress on a bacterial enzyme a new specific power of acting on this new substrate.

A similar speculation related to the "adaptive enzyme" concept is also advanced by Emerson (1945). He assumed that a disaccharide such as maltose can make a transmittable print on a gene which then conveys this to a protein in a manner complementary to that of the gene yielding a specific enzyme. The gene then constructed on this template should continue to produce the enzyme, even in the absence of maltose. This is another version of the "adaptive enzyme" concept which will be discussed below.

**b. Consideration of the Adaptive Enzyme Concept with Respect to Antibody Production.** The basic weakness in the theory proposed by Burnet, *et al.* would seem to lie in the fact that it is patterned after a concept which in itself is based on inadequate experimentation and interpretation of the factors controlling the assumed production of, or the increment in, the so-called "adaptive enzymes." As discussed previously (Sevag, 1946), the analogy between the claimed mechanism of adaptive enzyme formation and that of antibody production does not appear to be valid. Burnet, *et al.* postulate that the antigen molecule modifies a cellular proteinase which then is capable of synthesizing an antibody molecule. That is, the proteinase adapts itself not to metabolize the antigen but to synthesize antibody, a basic difference from the assumed acquired ability of adaptive enzymes to metabolize the specific substrate. Another noteworthy difference between antibody producing "adaptive proteinase" and "adaptive enzymes" is the duration of the former for the lifetime of the host and the inheritability of this character from parent to daughter host cells. In contrast, the lifetime of an adaptive enzyme is postulated to be limited. That is, it is formed when substrate is present and fails to function or form when the substrate in question is removed. "Adaptive enzyme" and "adaptive antibody" production concepts would have been more comparable if it could have been demonstrated that once the antibody producing enzyme system is born in a host, it could adaptively function to produce more antibody, provided the same antibody is introduced from time to time into the system which has ceased to produce measurable antibody. The assumed permanency of the antibody synthesizing

modified proteinase requires that this be a fact demonstrable in all cases. The proponents of the "adaptive enzyme" concept assume the existence of a *preenzyme* (Monod, 1942, 1944, 1945, 1947; Lwoff, 1946) with which a certain substrate combines and liberates the "adaptive enzyme" (discussed further below). On the basis of a complete analogy between Burnet's theory and the above theory of adaptive enzymes, it is to be noted that there does not seem to exist such a preenzyme, to adapt itself to synthesize antibody in contact with newly introduced antibody. The results of experiments have shown that the reinjection of antibody into a previously immunized host from which the antibody is derived, does not stimulate or initiate the production of more of the same antibody.

**c. Adaptive Enzymes—A Consideration of Facts and Assumptions.** In a previous communication (Sevag, 1946), enzymatic, chemical and theoretical reasons were given to reject the concept that the "production of adaptive enzymes" involves the synthesis of a new enzyme protein. Let us summarize the reasons for the position we maintain.

*First:* The pertinent data show that the so-called "adaptive enzymes" were present as integral parts of the cells prior to coming into contact with the substrates in question. In the presence of large amount of substrates, the necessary factors seem to be optimal for the maintenance or the demonstration of a measurable activity of the enzyme. The substitution of other structurally related substrates temporarily reduces the activity of the "adaptive enzyme" to a minimum, but does not abolish it. The reversal of the reduced activity to optimal activity with the return of the particular substrate to the metabolic environment has been claimed to be the production of "adaptive enzymes." The restoration of the activity to metabolize a substrate is also accomplished when the cells are grown either in the absence of that particular substrate, or also in the absence of a different substrate whose metabolism exercises a deleterious effect on the particular enzyme (Sevag and Swart, 1947). But under no conditions is the particular enzyme completely eliminated.

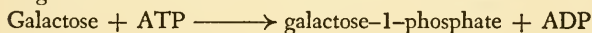
*Second:* "Adaptive enzymes" are formed only with those substrates which are configurationally related to a substrate which is more actively metabolized than others. This indicates a "master-key enzyme" for a group and not a specific enzyme for each substrate. It also means



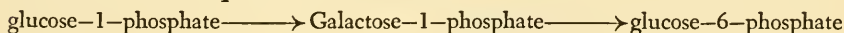
differences in the rates of the metabolism of various structurally related substrates. There is no evidence as to the presence of individual enzymes for each of the class of substrates which possess the same enzyme specific active group. Thus, according to the formulation of Weidennagen (1940), "there is no special key for each lock but a master key for a group of locks." Glucose, mannose, fructose, etc.; arginine, creatine, etc. possessing, respectively, the same configuration, would be metabolized by the same enzyme. Several dipeptides composed of different amino acids are hydrolyzed by Anson's crystalline carboxypeptidase, etc. In this connection the concept of Monod (1943, 1944, 1945, 1947) is of interest. According to him, all the adaptive and constitutive carbohydrate-attacking enzymes in bacteria may depend on a common mechanism of synthesis, or, more precisely, on a common precursor. The mutual-exclusion effect results from a competitive interaction of the substrates for the *preenzymes* or the common precursor. This concept likewise agrees with our view that under the influence of a substrate the synthesis of a new specific enzyme protein does not occur.

On the other hand, Spiegelman, *et al.* (1947) postulate that enzyme adaptation involves protein modification requiring energy-yielding metabolic reactions. Such assumptions cannot be accepted as valid until the postulated pre-protein and the enzyme derived therefrom are characterized, and the energy relationships of the chemical reactions leading from the former to the latter are established. The principal justification for the claim of the formation of "adaptive enzymes" seems therefore to lie in the fact that the enzyme activity for a certain substrate (for example, galactose)\* disappears or is greatly diminished

\*On the basis of the results of recent studies we are now able to gain an insight into the mechanism of the fermentation of galactose by yeast. Various steps involved in galactose fermentation are described as follows:



I



II

III

According to Wilkinson (1949) the first step (reaction I) in galactose fermentation by Dutch Top yeast is the transference of the terminal phosphate group from adenosine triphosphate (ATP) to galactose to give galactose-1-phosphate. (There was no evidence for the accumulation of galactose-1-phosphate during the pre-adaptive period.) The enzyme catalyzing this reaction is activated by  $\text{Mg}^{++}$  and by cysteine, and is named galactokinase. Since galactose is fermented via fructose-1,6-diphosphate, the question of how the galactose-1-phosphate is converted to glucose-1-phosphate (reaction II) arises. According to Caputto *et al.* (1950), the reaction II



when it is replaced by a more actively metabolizing substrate (for example, glucose). The increase in galactose fermenting activity with the elimination of the fermentation of glucose is explained by assuming that the common preenzyme is adapted now for one substrate and then for another. This does not appear to offer sufficient grounds for a theory of the production of a new enzyme protein from a common protein when it is in contact with each member of a group of related substrates. The application of the enzyme precursor idea to such problems seems to exaggerate the significance of such changes.

In an actively metabolizing environment with or without outside nitrogen source, an enzyme can be reversibly changed to an inactive form. The restoration of activity when in contact with substrates or in their absence in no way justifies the assumptions that the inactive precursor form originated first during the cytoplasmic synthesis of the protein. The fact that the active enzyme can assume an inactive or weakly active state when a substrate (galactose) is eliminated, or when its metabolism is followed by the metabolism of another substrate, shows clearly that the enzyme which was once active, could assume an inactive form, and therefore justifies the belief that the active form of the enzyme is synthesized first. There seems therefore no experimental basis for hypothesizing the existence of enzyme precursors as being synthesized prior to the active enzyme (see further a succeeding section).

*Third:* Cells do not manifest the quality of producing a new, "adaptive enzyme" for a substrate which does not possess an enzyme-specific reactive group common to a substrate which is normally readily metabolized by a given cell. A cell cannot be forced to produce an enzyme to metabolize a substance for which there is no genetic provision in its species specific physiology and economy. *Staphylococcus aureus*, for example, lacks the ability to metabolize formate in a manner similar to that exercised by *E. coli*. *Staphylococcus* lacks the genetic ability to produce the formic hydrogenylase. *Hemophilus influenzae* has not been shown to adapt itself to synthesize the porphyrin molecule

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involves a Walden inversion of C-4 and is catalyzed by an enzyme which they call "galactowaldenase." The coenzyme of this reaction has been isolated and characterized as uridine-diphosphate-glucose (UDPG). This coenzyme has been found in animal tissues and in yeast not adapted to galactose.  $\alpha$ -1,6-Glucosediphosphate, discovered and isolated by Cardini *et al.* (1949), has been found to function as coenzyme for phosphoglucomutase (reaction III).

and coenzyme I no matter how long it may be in contact with them. Pneumococci do not contain catalase, and are incapable of synthesizing an enzyme no matter how long they remain in contact with catalase or with non-toxic concentrations of hydrogen peroxide as substrate for catalase. *Staphylococcus aureus* is incapable of synthesizing nicotinamide and thiamine despite the fact that they are capable of incorporating them into their metabolic system.

The assumption of Spiegelman (1946) that there appear endogenous sources of protein, which are available for transformation into enzyme protein does not therefore appear to be a valid and experimentally tenable conception. His assumption also that when a cell is *forced* to form a new enzyme it may draw upon existent enzymes as a source of protein has been shown to be an impossibility in those bacterial cells where the required particular enzyme (so-called "adaptable" enzyme protein) is not in existence to start with. The above cited facts as examples of generally occurring events contradict these assumptions that an organism can synthesize a new enzyme under pressure.

Under any circumstances, if an adaptive enzyme formation is a reality, it is essential that its characteristics be determined not by an increment in a common type of a metabolic effect, but by unequivocal classical methods of chemistry and immunology. The application of the following two critical tests seems to be essential:

- (1) That there be an absolute qualitative and quantitative difference in the amounts of "galactose apoenzyme" (Spiegelman, Reiner, and Morgan, 1947) in adapted and non-adapted yeasts; and
- (2) That the assumed presence of this adapted galactose apoenzyme can serologically be demonstrated in the galactose fermenting adapted and not at all in the non-adapted yeast cells.

The latter method of analysis should particularly be useful in view of the enormous number of experimental facts to show that  $\alpha$ - and  $\beta$ -configurations, d- and l-activity,  $-\text{COOH}$  and  $-\text{CONH}_2$ , etc. closely related groups present in antigens can be finely differentiated. If the observed increment in the fermentation of galactose is due to a *de novo* synthesis of a species of an enzyme protein, it should be possible to demonstrate this synthesis in the above manner. The presence of even a few molecules of this enzyme protein in a non-adapted cell means a genetic provision to synthesize this enzyme. A measurable increment

in this respect in an "adopted" cell is merely a quantitative change and, therefore, does not represent theoretical significance.

**d. Consideration of the Primary Role of Antigen in Antibody Production as Postulated by Burnet, *et al.*** Burnet, *et al.* postulate that antigens taken into the antibody forming cells lastingly modify cellular proteinase units which then synthesize antibodies specific to antigens. The claimed lasting character and the inheritability from parent to daughter cells of this modification implies, in the light of the present concept of the role of genes in the synthesis of specific enzymes, that antigens bring about an inheritable variation in the genes. This variation is not a degradative mutation but a building-up process since the specific enzyme system not only is still capable of synthesizing the total quota of normal but also the new species specifically related antibody globulins. This assumption conflicts with the well established genetic characteristics of species.

Known facts instruct us that a cell may undergo the following three types of genetic changes:

- (1) Chromosomal changes and gene mutations. Such directed changes have not been demonstrated in antibody formation against foreign proteins.
- (2) A building-up process which requires the incorporation and inheritance of genetic factors acquired by a cross-fertilization (Lindegren and Lindegren, 1945), or, in an asexual (?) cell (e.g., bacteria), by the incorporation into the recipient recessive cell of cytoplasmic material or of a catalyst of genetic nature from another strain of higher order but of the same species (Avery, *et al.* 1944). Recombinations might also result in degenerative changes depending on the nature of recombining factors.
- (3) Degenerative mutations by which a cell undergoes hereditary losses as the consequence of the action of toxic agents, as observed in the development of resistance to drugs (Sevag, 1946). The hereditary cytoplasmic changes in the enzymatic constitution of yeast (Ephrussi, *et al.*, 1949; Slonimski and Ephrussi, 1949) are clearly degenerative changes. The changes in the antigenic types in *Paramecium aurelia* (Sonneborn, 1949) may also be interpreted as degenerative changes. In this case, every potentiation of an antigen results from or is associated with the suppression of another.

According to the first process, only species specifically related strains of cells cross-fertilize to produce a new strain. No case is known which shows genetic cross-linkings between cells not related species specifically. It would therefore seem improbable that thousands of proteins and thousands of artificial antigens species specifically foreign to the host undergoing immunization are capable of genetically cross-linking with the genes of the host to bring about a building-up process in the antibody producing cells as implied by the theory of Burnet, *et al.*

If the life-long immunity to measles and yellow fever viruses persists long after these agents are eliminated (assuming that this is an absolutely proven experimental fact), one may, perhaps, indulge in speculating that these viruses function as "quasi-genetic factors" related species specifically to the globulin synthesizing cells of the host by being derived from them so as to offer a support for the implications of Burnet's theory. However, there are as yet no traces of experimental data that smallpox, measles and yellow fever viruses bear species specific relationship to the host cells involved in antibody globulin synthesis. On the other hand, the generally supported idea emphasizes the fact that the observed life-long immunity to these viruses may be due to their multiplication in the host as variants deprived of their properties to cause observable pathological symptoms, as for example, the cow-pox virus, probably arising as a mutation of human smallpox, modified rabies virus produced by brain-to-brain passage in rabbits, and yellow fever virus strain 17D which was evolved by hundreds of passages in tissue culture in media containing embryonic tissue from which neural tissue had been removed. The resulting virus has lost its neurotropic character and has been widely used for human immunization (Theiler and Smith, 1936).

Reference can be made also to six mutant strains of tobacco mosaic virus each producing characteristic leaf symptoms, and possessing different serological properties and amino acid composition (Knight, Stanley, 1941; Ross, 1941, 1942). These facts would fall in line with our theory of *Immuno-catalysis* that viruses, like other antigens, function as catalysts in producing specific antibodies. In this role, they do not produce a permanent genetic change in the globulin synthesizing cellular enzyme system, but direct certain steps in globulin synthesis to yield antibody globulin. This directive influence continues to function so long as antigen persists (Libby, 1947) as a component



of the complex enzyme system involved in globulin synthesis. Antigens thus function as extraneous catalytic factors, and not as specific modifiers of the cellular proteinases.

Dougherty, White and Chase (1945) compared the antibody content of normal and malignant lymphocytes. Hemolytic filtrate of *Staphylococcus aureus* was used as antigen in the immunization of mice. Tumors were transplanted into mice in the middle of, before and following the courses of immunization lasting from 15 to 30 days. Extracts of normal and malignant lymphocytes, and blood sera were assayed for their antihemolytic antibody titers. They reported that the growth of an antibody-containing tumor transplant in normal mice was accompanied by the development of antibody-containing malignant cells. The normal lymphocytes of the host animal receiving this transplant likewise contained antibody, "As the growth of the tumor proceeds, increasing number of antibody-containing lymphocytes are formed. Thus, the total quantity of available antibody in the lymphoid structure is dependent upon the number of antibody containing lymphocytes. This type of antibody production, i.e., multiplication of pre-existing cells containing immune globulin, may be a prominent mechanism of antibody production in the normal organism."

On the basis of the above quotation one may be tempted to postulate that the lymphocytes have acquired the ability to synthesize antibody in the absence of antigen. However, there is no statement concerning the question of the number of transplants one can make before the antibody formation ceases. In conversation, Dr. A. White informed me that the third or fourth transplant ceases to show the presence of antibody. It would thus indicate that successive generations of lymphocytes are non-homogeneous with respect to their content of antigen. In the third transplant only a few lymphocytes will contain antigen. That is, the antigen will be so diluted by this time that it can no longer produce a measurable amount of antibody.

**e. Consideration of Antigens as Toxic Agents Causing the Mutation of Globulin Synthesizing Enzyme System.** Another point which may be worthy of consideration is the question of whether or not antigens function as toxic agents or inhibitors of the enzyme system involved in the synthesis of globulins. If they function as toxic agents one may assume that they bring about, as stated above, the second type of change in a cell which is of degradative nature. Since this type of



change, unlike the changes ensuing from cross-fertilizing genetic changes, is invariably brought about by agents genetically, or species specifically, not related to the cells undergoing this change, and since antigens are foreign to the host undergoing immunization, one may postulate similarities between the action of antigens and mutagenic chemical agents.

Such a change of degradative nature could be expected to yield mutant cells which, one may assume, produce specific antibodies as abnormal by-products. As in all degradatively produced mutant cells, such a postulated change in host cells would be expected to be of permanent and inheritable character, and would, superficially, support the idea of life-long lasting immunity to certain viruses claimed by Burnet, *et al.* and implied by their theory. Since, however, immunity to the dominant number of toxic and non-toxic antigens is of temporary nature, one must assume that antibody producing cells or their enzymes do not undergo a degradative mutation preceding or concomitant with active immunization. Furthermore, toxic agents which induce mutations, are known to suppress or interfere with the metabolism or synthesis of specific cell metabolites or components. In contrast, immunity to antigens is accompanied by hyperglobulinemia and not by hypoglobulinemia, indicating that antigens do not suppress the synthesis of globulins.

Certain antigens produce toxic effects and pathological conditions in a host followed by antibody production or immunity. Non-antigenic drugs or toxic agents also produce noxious effects on cells (for example, inhibition of enzyme activities) resulting in the development of resistance to these effects. Unlike the latter effects, certain antigenic toxins, for example, produce their effect functioning as enzymes and destroying the host tissue components, e.g., lecithinase, proteolytic, necrotic and hemolytic actions of bacterial products and snake venoms. However, the host system is not known to develop a non-immune resistance\* to these toxins in a manner comparable, for example, to the resistance manifested by a bacterium to a drug. Bacterial drug

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\*If a resistance in a host can be shown to have resulted from the action of the toxins of infectious agents, it is not improbable that the action of the toxins, like those of the antibacterial drugs (Sevag, 1946; Sevag and Gots, 1948; Steers and Sevag, 1949; Sevag and Steers, 1949), have permanently abolished the receptor sites of the host tissues. This would result in a life-long resistance to the toxins of the infectious agents concerned.

resistance does not involve a continued production of an antibody-like substance capable of combining with a drug to neutralize its toxic effect. On the other hand the only resistance a host develops to an antigenic toxin is believed to be by means of the production of specifically combining and neutralizing antibodies during a limited period.

Consideration of the above discussed questions seems to show that the phenomenon of immunity is conditioned by the presence of antigenic units but that this conditioning disappears when the antigenic units are eliminated from the host system.

## 6. On Jordan's Autocatalytic Theory of Antibody Formation

Jordan (1944) has proposed an autocatalytic theory of antibody production. According to this theory, the multiplication of a gene or a virus only in the presence of itself, is a basic autocatalytic phenomenon in all living processes including the formation of antibody. The molecules, or fractions of molecules which possess the ability for parallel orientation and are capable of multiplication are identical and exhibit affinities for each other. A molecule  $M$ , which is capable of multiplication, may be composed of  $\mu_1$ ,  $\mu_2$ ,  $\mu_3$  fractions each of which possesses the property of attracting others to itself. These molecular fractions *per se*, in solution, by themselves possess very little tendency to combine with each other; they do so, however, when  $M$  is present forming another molecule identical with  $M$ . The attraction among the fractions  $\mu_1$ ,  $\mu_2$ ,  $\mu_3$  is attributed to resonance of the fractions. That is, two substances are said to attract each other through resonance when they possess groupings identical or nearly so. Jordan had earlier expressed similar views concerning this question which had been denied by Pauling and Delbrück (1940) on grounds that the resonance energy would be so small as to be ineffective. Reasoning in similar fashion, Jordan (1940) had postulated that antibodies and specific antigens are identical, a postulate which is related to the concept of Büchner (1893). This likewise has been refuted by the chemical-immunological evidence supplied by Haurowitz, *et al.* (1942). The latter investigators using iodo-proteins, bromo-protein and arsonil-azoprotein as antigens showed that none of the antibodies contained the

determinant group of the antigen or a serologically related group in the respective antibody molecules. The concept of the identity of antigen and antibody postulated by Jordan must be rejected also for genetic reasons, and for reasons that an antigen is antigenic by virtue of being species specifically unrelated to the antibody globulin elaborated by the immunized host.

Jordan (1944) assumes that antibody formation does not take place during a period of from six to 48 hours following the injection of antigen. This assumption is to be denied for it has been observed (Hamburger, 1902; Oerskov and Anderson, 1938; Ramon, 1928, etc.) that the production of antibody can be demonstrated three to four hours after the injection of antigen. On the basis of this assumption Jordan postulates that antigens initially produce only small amounts of antibodies, and that the subsequent increase in the amount of antibody results from an autocatalytic process mediated by the antibody molecule itself. As stated above, a measurable amount of antibody has been demonstrated a few hours after the injection of antigen. There is no doubt that non-measurable amounts of antibody must have been synthesized at a still earlier period, indicating a rapid output of antibody by a process which, in our opinion, is possible if antigen functions as a catalyst.

The autocatalytic concept of Jordan implies that the antibody production should be a continuous process. A second implication of Jordan's concept is that the introduction of an antibody obtained from an actively immunized animal into another animal of the same species should autocatalytically produce new antibody in amounts many-fold greater than the amount introduced. The production of antibody postulated in this manner in a normal individual would be comparable to the production of an infection in a host by an infectious agent derived from a disease bearing host. Jordan considers this possibility in the above described manner, but was unable to cite a single experimental observation. As is well known, passive immunizations are of very short duration, and the experiments of Heidelberger, Schoenheimer and their associates (1942), as discussed in a preceding section, have shown that an antibody derived from a rabbit and introduced into a normal rabbit enters into metabolic reactions resulting in its disappearance.

## 7. The Concepts of "Proteinogen" and Enzyme Precursors Considered in the Light of the Specificities of Antigens and Antibodies and Their Digestion Products

Northrop (1946, 1948) has conceived the idea that normal proteins, enzymes, antibodies, viruses are derived from a "proteinogen" or "ur-protein" by a purely catalytic, or autocatalytic reaction which does not require energy. The synthesis of the "proteinogen" molecule, requiring energy from another source, is, likewise, assumed to be autocatalytic. The proteinogen molecule possesses the general chemical structure characteristic of the species. The formation of the above mentioned biologically and chemically specific proteins from the master proteinogen molecule is considered as analogous to the formation of pepsin and trypsin from their respective precursors. Assumption is also made that by such transformations from proteinogen to specific proteins the latter acquire specific enzyme and immunological properties.

If we understand Northrop's postulates correctly, they imply that the derivation of the specialized functions of various proteins found within a kind is associated, or brought about, by an autocatalytic reaction which is capable of converting proteinogen molecules into many different species of protein molecules. On this basis, enzymes, such as lysozyme, d-ribonuclease, cytochrome c, with molecular weights of about 15,600, myoglobin (mol. wt. 15,900), lactalbumin (mol. wt. 17,400) etc. which are structurally, enzymatically and serologically different from species specifically related proteins have acquired these specific properties during their autocatalytic geneses from proteinogen molecules. Since, as stated by Northrop, the molecular weight of the proteinogen molecule is equal to or greater than that of the protein derived, and since the autocatalysis in these conversions does not require energy, it must be assumed that: (a) there must be as many specific proteinogens as there are specific proteins. For example, molecular weights for certain specific proteins are: pepsinogen, 42,000; pepsin, 38,000; chymotrypsinogen and chymotrypsin, about 40,000; and trypsinogen and trypsin, 35,000; (b) it must be assumed that the master proteinogen molecule must have a molecular size larger than the largest specific molecule found in a species, so as to be autocatalytically convertible to all molecular sizes; or (c) there must be a proteinogen



molecule which autocatalytically can be polymerized or aggregated to yield the largest molecular species one can find in a species.

**a. Consideration of Enzyme Precursors.** Pepsinogen is changed into pepsin under the following conditions: (a) by hydrogen ion on the acid side of pH 6.0 (maximal activity at pH 2.0); (b) by addition of pepsin; or, (c) increasing the salt concentration to increase the rate of conversion. This change from pepsinogen to pepsin in each case is associated with the dissociation of a polypeptide, with a molecular weight of 6,000, which is capable of recombining with pepsin at pH 5 to 6 to form a dissociable pepsin-inhibitor complex. In this combination the activity of pepsin is blocked but not destroyed. On long standing with pepsin between pH 2.0 and 5.0, the inhibitor is destroyed. The pepsin inhibitor has exposed basic groups.

The change from chymotrypsinogen to chymotrypsin by slightly acid solution, or by trypsin, would appear to be the result of a limited hydrolytic reaction without any apparent effect on the molecular weight of the inactive protein. According to Northrop (1937), the chymotrypsin molecule contains five amino groups\* more than chymotrypsinogen, indicating the opening of a peptide ring. There is also an associated shift in isoelectric point from pH 5.0 to 5.4. No change was observed in elementary analysis, and the tyrosine plus tryptophane content.

In the change from trypsinogen into trypsin, no measurable increase in amino groups was detected. As suggested, it might be that the hydrolysis of a peptide link, too small to be detected, might have taken place. The change from trypsinogen to trypsin takes place at pH 7.0 to 9.0 without the aid of any outside activator, at pH 3.0 to 4.0 by means of a mold kinase (Kunitz, 1938), in the presence of enterokinase at pH 6 to 9.0 where spontaneous activation of trypsinogen occurs readily, or, simply, in the presence of calcium ion at pH 8.0 and 5°C (Kunitz, 1945). Under this latter condition, trypsinogen is quantitatively converted into trypsin without the formation of "inert protein" formed under other conditions.

In the above cited typical examples of enzyme precursors which are studied more completely than any other crystalline enzymes isolated, the change from inactive to active form involves treatments which are

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\*Amino nitrogen as per cent of total nitrogen: chymotrypsinogen, 4.7; chymotrypsin, 6.0 (Northrop, Kunitz and Herriott, 1948).



found not to cause deep structural variation. One may therefore, perhaps, ask the question of whether or not these changes can be taken as proof that these enzyme precursors are really the forms which are synthesized first. It would seem that the inactive forms could easily be assumed to have been derived from the active forms under *in vitro* or *in vivo* environmental influences.\* The ease with which pepsin can reversibly combine with pepsin inhibitor recovered from pepsinogen complex ( $\text{Pepsin} + \text{pepsin inhibitor} \rightleftharpoons \text{"pepsinogen"}$ ), and the ease with which both chymotrypsinogen and trypsinogen are changed to their respective active forms with reagents such as calcium ion, or, spontaneously, at neutrality, permits, as an alternative, the assumption that the enzyme molecule is synthesized first and subsequently converted into certain reversibly inactive forms. As will be discussed later in this work, serum proteolytic enzyme which, among other proteins, also digests fibrin clot exists in an inactive form in normal serum. The treatment of serum with chloroform, ether, ethyl alcohol, etc., or dialysis, liberate the active enzyme; the enzyme may also be liberated spontaneously, on standing in the cold. Thus, it is most likely that these treatments dissociate a substance from an inactive complex which blocked the activity of the serum protease.

Northrop (1946) mentions the serological behavior of the precursor and the respective active enzyme as proof that the latter is derived from the former, or that a protein may be formed by an autocatalytic re-

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\*One must ask the question of whether chymotrypsinogen is a precursor of the chymotrypsins or an artifact derived from native chymotrypsin as the result of the conditions used for their isolation from the source material. Similar question may be raised with respect to the relationship between trypsinogen and trypsin. In relation to these questions we must be aware of the fact that there is no evidence that chymotrypsinogen (and trypsinogen) *per se* exist in the pancreatic tissue. All that we know is that they are obtained from extracts made by means of strongly acid, 0.25N  $\text{H}_2\text{SO}_4$ , solutions at a temperature of 5°C. After several fractionations from solutions on the acid side, a crystalline material from a solution at pH 5.0 is obtained. A solution of these crystals on adjusting to pH 7.6 with a trace of trypsin, yields the crystalline chymotrypsin (Northrop, *et al.* 1948).

The intriguing question is the nature of the possible effect of the cold strong acid on the raw source of these enzymes. Is it not possible that under the above preparative conditions these enzymes undergo dehydration of the free amino and carboxyl groups yielding chymotrypsinogen (and trypsinogen) which reverse to the active forms spontaneously in weakly acid or alkaline solutions with or without the aid of activating agents?

While correcting the page proof, S. D. Elliott (J. Exp. Med., 92:201-218, 1950) reported that the maximal production of the precursor of streptococcal proteinase occurred only in slightly acid, and none in neutral or alkaline environment.

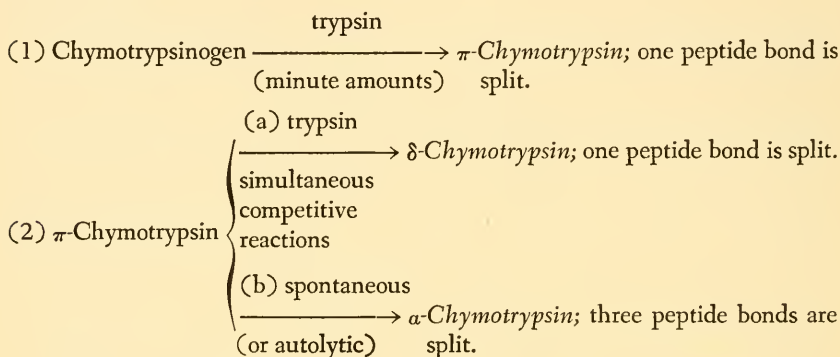
action from another protein. Ten Broeck (1934) reported that guinea pigs receiving chymotrypsinogen were sensitized against the so-called precursor itself but not against chymotrypsin, and guinea pigs receiving chymotrypsin were sensitized against itself, but not against chymotrypsinogen. But, he also reported that in some cases there were cross-reactions, particularly between the chymotrypsin and chymotrypsinogen, indicating a close serological relationship between the inactive and active proteins.

In the absence of minute amounts of trypsin, the change from chymotrypsinogen to chymotrypsin may occur spontaneously, slowly at pH 5.0, and faster in slightly acid or weakly alkaline solutions. The change from trypsinogen to trypsin is reported to occur in the presence of calcium ion and at pH 7.0 to 9.0 without the aid of any outside activator. Under these conditions, the increase of five free amino groups in chymotrypsin does not appear to indicate an opening up of stable peptide linkages as would result from the characteristic action of proteolytic enzymes. These changes may involve salt-like reactions with basic or acidic groups, or result perhaps from a reaction involving hydrogen bonding. Under such conditions a serological difference between the two forms of protein is conceivable (see Pauling in Landsteiner, 1945). As such, chymotrypsinogen, for example, could be a derivative of chymotrypsin and not a precursor. In relation to this, it may be pointed out that an antigen can be modified in similar manner to yield an immunologically different protein derivative. Landsteiner, *et al.* (1932) reported a serological difference between an antigen with a terminal  $-\text{COOH}$  group and that having  $-\text{CONH}_2$  as terminal group instead. Or immune sera prepared with a methyl ester antigen precipitated the homologous azoprotein but not that made from the parent, p-aminobenzoic acid. If the ester was hydrolyzed by gentle alkaline treatment of the azoprotein a strong reaction with serum for p-aminobenzoic antigen (Landsteiner, *et al.* 1927) took place. Chow and Goebel (1935) showed that acetylation of the amino groups of an antibody abolished its reactivity with a specific polysaccharide. Conversely, the esterification of the  $-\text{COOH}$  group of the polysaccharide deprived it of its reactivity with the native antibody. Saponification of the esterified carbohydrate restored the activity to react with the specific antibody.

In the above cited instances with the inactive and active forms of

enzymes, and in the latter instances with antigens and antibodies, we are observing reactions with superficial groups and not deep seated chemical changes in the structure of protein molecules. In immunologic respects, the case of trypsinogen and trypsin, or chymotrypsinogen and chymotrypsin, does not appear to deviate from the above basic processes.

**b. Derivatives of Chymotrypsin and Their Properties.** In a comprehensive investigation, Jacobson (1947) reported that the tryptic activation of chymotrypsinogen at 0°C consists of the following transformations:



*Note:* In connection with the above spontaneous conversion of  $\pi$ -chymotrypsin to  $\alpha$ -chymotrypsin it may be recalled that Kunitz and Northrop (1935) had found that chymotrypsinogen spontaneously (without the minute amounts of trypsin) undergoes a change, leading to the formation of chymotrypsin. There was no marked pH optimum for this change, but it occurred faster in weakly acid or alkaline solutions independent of the effect of trypsin.

$\pi$ -Chymotrypsin is an hitherto unrecognized chymotrypsin, and is not isolated.

$\delta$ -Chymotrypsin is likewise unrecognized hitherto and is not crystallized. *Specific activities:* Jacobson reported that the specific activity of

$\pi$ -Chymotrypsin is 2 to 2.5 fold  $>$   $\alpha$ -chymotrypsin, and that of  $\delta$ -chymotrypsin is 1.5 fold  $>$   $\alpha$ -chymotrypsin.

It is most interesting to note that in the conversion of inactive chymotrypsinogen into the above three chymotrypsins, the splitting of one "peptide bond" yields the most active enzyme,  $\pi$ -chymotrypsin, and that  $\alpha$ -chymotrypsin, resulting from the splitting of *five* (?) "peptide bonds" in chymotrypsinogen, is the least active form.

According to Jacobson, the number of titratable groups per  $\alpha$ -chymotrypsin molecule is greater by six to nine than that per chymotrypsinogen molecule, indicating a probable hydrolysis of four peptide bonds during the conversion of chymotrypsinogen to  $\alpha$ -chymotrypsin. He considers several possibilities to support or refute his assumption that the above transformations involve the splitting of peptide bonds.

He considered for chymotrypsinogen a protein structure as formulated by Mirsky and Pauling (1936). According to these authors, the native globular unconjugated proteins consist of an uninterrupted polypeptide chain which is continuous throughout the molecule. The polypeptide chain folds into an uniquely defined configuration. This configuration is maintained by means of hydrogen-bonding between the peptide nitrogen and oxygen atoms and also between the free amino acid carboxyl groups of diamino and dicarboxylic amino acids. In the case of chymotrypsinogen, the hydrolysis of peptide bonds, leading to chymotrypsin with six to nine titratable groups more than the original molecule, must occur near the ends of the peptide chain. The hydrolysis of certain terminal amide bonds by trypsin was taken into consideration in view of the fact that trypsin is able to hydrolyze the amide bond in benzoyl-glycyl-arginamide, benzoyl-arginamide, benzoyl-glycyl-lysine-amide and benzoyl-lysine-amide (Bergmann, *et al.* 1939). Such a hydrolysis of a protein should yield ammonia. The finding of Butler (1941) that at the maximum activation by trypsin 1  $\text{NH}_3$  per 12 chymotrypsinogens is produced, and no increase in  $\text{NH}_3$  took place after 66 hours, was not considered of any significance by Jacobson. It would, however, seem that the ease with which the amides are hydrolyzable might be in line not only with the catalytic conversion of chymotrypsinogen into chymotrypsin by trypsin, but also, particularly, with the spontaneous (without the presence of minute amounts of trypsin) change of chymotrypsinogen into chymotrypsin as has been observed.

According to Jacobson, the splitting of a terminal amide bond from chymotrypsinogen does not occur to any significant extent during the tryptic hydrolysis leading to  $\pi$ -chymotrypsin. The splitting off of an amino acid  $\alpha$ -amide during the autolytic (or spontaneous) conversion of  $\pi$ -chymotrypsin leading to  $\alpha$ -chymotrypsin is not impossible if such bonds exist in chymotrypsinogen since  $\alpha$ -chymotrypsin is reported to possess such an activity (Bergmann and Fruton, 1938; Fruton and



Bergmann, 1939), however, Butler's results do not indicate that the hydrolysis of amide bonds occur to any significant extent during the activation process. In view of these experimental material, Jacobson does not seem to favor, on the basis of the formulation of Mirsky and Pauling, that chymotrypsinogen contains or consists of one uninterrupted peptide chain. Assumption is made that if proteins contain several peptide chains, the breaking of about four peptide bonds in chymotrypsinogen with the result of an increase of six to nine titratable groups when  $\alpha$ -chymotrypsin is formed, may be explained. This could result from the proteolytic rupture of peptide bonds in the peptide chain or chains of chymotrypsinogen and of the proteins (one of which is  $\pi$ -chymotrypsin) which are intermediaries of chymotrypsinogen and  $\alpha$ -chymotrypsin, without necessitating the formation of non-protein-nitrogen. This, however, leaves unexplained the origin and nature of 2.7 per cent non-protein-nitrogen formed during these reactions, part of which at least can be accounted for as ammonia nitrogen as Butler (1941) has found.

Jacobson arbitrarily chooses a molecular weight of 36,000 (36,700 by Brand and Kassel, 1941) for chymotrypsinogen. Since he found the production of 2.7 per cent non-protein-nitrogen during the conversion of chymotrypsinogen into  $\alpha$ -chymotrypsin, the molecular weight of the latter was calculated to be at least 35,000. These values are basically different from those of 32,000 to 36,000 for chymotrypsinogen, and 40,000 for  $\alpha$ -chymotrypsin as measured by Kunitz and Northrop (1935), and Kunitz (1939). Consequently, Jacobson's chymotrypsin would appear to be a derivative of diminished molecular weight, and that of Kunitz and Northrop a chymotrypsinogen derivative of about 11 per cent increased molecular weight. This discrepancy may, however, be due to the inconstancy of the molecular state of the active enzyme.

In connection with the above observations of Jacobson, it is to be noted that earlier Kunitz (1938) had prepared crystalline  $\alpha$ - and  $\gamma$ -chymotrypsins from chymotrypsin. These two proteins did not differ in activity from the parent chymotrypsin of 40,000 molecular weight. The molecular weights of  $\alpha$ - and  $\gamma$ -chymotrypsins were reported to be, respectively, 27,000 and 30,000. It is interesting that a non-essential part or component corresponding to a molecular weight of 10,000 to 13,000 can be split off the chymotrypsin molecule (or complex) with-



out affecting the degree of enzyme activity and immunological specificity (Northrop, *et al.* 1948). It would be interesting to know how far one can reduce the molecular size of the smallest of the three chymotrypsins without affecting its serological and enzymatic properties.

c. **Virus-Host Relationship.** The experimental demonstration of the independence of the biophysical and serological specificities of virus preparations derived from different species of hosts may, at present, for technical reasons, be difficult to achieve, particularly with animal viruses of greater complexity and particle size which have not lent themselves to purification, for example, by crystallization.

The failure to separate the host components from such virus preparations may easily give rise to assumptions that these viruses carry the specificity of tissues from which they are derived. It has been reported (Knight, 1946), for example, that the virus of influenza contains a specific normal component which is characteristic of each individual host from which the virus is obtained. However, this component exhibits a serological specificity distinct from that of the virus. On the other hand, similar studies with plant viruses which are simpler in composition and are obtainable in crystalline form have shown the biophysical and serological individuality of the viruses irrespective of the plant hosts in which they are multiplied (Bawden and Pirie, 1944, 1946; Gaw and Stanley, 1947; Malkiel, 1947). It has been found that the purified preparations of tomato bushy stunt virus derived from the leaf sap and the fibrous residues of infected tomato plants did not differ in any significant manner. Purified tobacco mosaic virus and the rib-grass strain of tobacco mosaic virus, in these respects, showed identical properties. Malkiel (1947) reported that there was no serological difference found for either tobacco mosaic virus or rib-grass virus when each was isolated from widely separated plant species. Immunochemical studies failed to indicate the presence of any normal host protein as a component of any of the virus preparations. These findings do not offer any support to the assumption that the viruses are autocatalytically derived from a normal proteinogen possessing the species characteristics of the host in which the virus multiplies.

And there are, at present, no data to indicate that normal host proteins or the viruses derived from the host can be modified chemically or otherwise, to show any serological interrelationship suggestive of the autocatalytic origin of viruses from the host proteins.

Any body of experimental data offered for the formulation of a concept dealing with the mechanism of the multiplication of a viral or bacterial parasite, and that of the synthesis of viral or bacterial proteins, must take into consideration the following well-known facts: (a) no living cell as yet has been found to synthesize a protein which is not species specifically related to itself; and (b) no living unit with distinct genetic makeup has been demonstrated to exist which is capable of directing, in accordance with the image of its own species specific characteristics, the synthetic facilities of another living unit belonging to an entirely different species.

**d. Structural Specificity of Polypeptides of Low Molecular Weight.** The above considerations suggest that structural specificity is introduced into protein molecules during their synthesis from smaller building blocks. What is more fundamental is the fact that enzymes of the smallest unit of molecular weight (15,000), that is the smallest unit which cannot reversibly be split, possess biological specificities. Polypeptides with a molecular weight of 6,000, such as trypsin and pepsin inhibitors, and d-glutamic acid polypeptide of *B. anthracis* possess specific serological (Ivánovics and Bruckner, 1937, 1940) and, other activities. Pepsin inhibitor, for example, specifically combines with pepsin, but has no demonstrable effect on the activity of crystalline trypsin and on the milk clotting activity of crystalline chymotrypsin or commercial rennet. Trypsin inhibitor isolated from trypsinogen crystals (inhibitor-free trypsinogen crystals have not been obtained, Kunitz and Northrop, 1936) inactivates trypsin, activates chymotrypsinogen to chymotrypsin, but has no effect on the milk clotting action of pepsin. These findings show that polypeptide molecules of 6,000 molecular weight show a high degree of structural specificity. Irrespective of their origin, these characteristics are in the polypeptide molecules, indicating that they are introduced there during their synthesis as independent units, or as parts of certain larger protein molecules, split therefrom, perhaps, by enzyme hydrolysis.

In this connection, it is of interest to note that Kunitz (1945, 1946, 1947) isolated a trypsin inhibitor of globulin nature from soy bean which formed a crystalline inactive complex with trypsin. Lineweaver and Murray (1947) isolated from egg-white an ovomucoid (mol. wt., 29,000) which caused 50 per cent inhibition of trypsin at equimolar concentrations. It would be of pertinent interest to learn if these latter

two inhibitors can be subjected to hydrolytic cleavage to yield polypeptides comparable with those isolated from pancreas and serum (Schmitz, 1938).

Landsteiner and Chase (1933) showed that the reaction between the antigen, sheep serum, and its homologous antibody could be inhibited by a readily dialyzable albumose obtained from the products of peptic digestion of the coagulated sheep serum antigen. Landsteiner (1942) reported also that the products of silk hydrolysis consisting of peptides having molecular weights from 600 to 1,000 were capable of inhibiting the reactions of precipitin sera for silk. From these results Landsteiner inferred that silk fibroin contains determinant structures of not more than eight to 12 amino acids. Holiday (1939) immunized rabbits with horse serum albumin which was purified by electrophoresis and shown to be homogeneous in the ultracentrifuge. The purified antigen was digested with 1/4000 parts by weight of pepsin at pH 2.0 for periods of five and 30 minutes.

In electrophoretic analysis the five minute digest, showed two components with mobilities different from that of the intact antigen, and by ultracentrifugation these components were inferred to have a size  $\frac{1}{4}$  that of the original molecule. The 30 minute digest showed the components to be of  $\frac{1}{8}$  the size of the intact antigen. The  $\frac{1}{4}$  size components showed practically undiminished precipitating activity, and  $\frac{1}{8}$  size components showed no precipitating activity but partially inhibited the reaction between the whole antigen and antibody. The  $\frac{1}{4}$  and  $\frac{1}{8}$  size digestion products would have molecular weights of about 17,000 and 8,500 respectively. Holiday concluded that at the stage of division into eight parts there is still evidence of affinity between the digestion products and antibody. In this connection it is interesting to observe that non-protein products formed by peptic digestion of ovalbumin, according to Tiselius and Ericksson-Quensel (1939) had an average molecular weight of 1,080 (see also Haugaard and Roberts, 1942). Winnick (1944) reported that partial hydrolysis products from the action on casein of chymotrypsin, trypsin, pepsin, ficin or papain yielded products with an average molecular weight ranging from 600 to 450. Serological activities of these products were not studied.

The above facts concerning the structural specificities of polypeptide molecules of as low a molecular weight as 600 to 1,000 seem to show

conclusively that these specificities are inherent in the reactions involved in their synthesis. It is difficult to conceive that a master proteinogen molecule can contain hundreds or thousands of specificities, and the cells of each organ or specialized structure of the body must all contain the same proteinogen molecule. Our existing knowledge does not lend any support to these assumptions.

e. **Does Antigen Function as Coenzyme in the Production of Antibody?** Northrop applies the proteinogen concept to the production of highly specific antibody proteins. He is of the opinion that antibodies are formed in a manner comparable to the formation of "adaptive enzymes." We have already discussed this process and concluded that it does not involve the synthesis of a new protein. The formation of an antibody, on the other hand, is a synthesis of a new protein as will be discussed below. What is more surprising is the analogy drawn between an antigen and a coenzyme; the former in the rôle of the latter is supposed to be capable of autocatalytically modifying serum proteins so that an antibody, instead of the normal serum protein, is formed. The hypothetical implications of the experiments of Pauling and Campbell (1942) may have been held in view in making this postulate. These authors reported the manufacture of "antibody" *in vitro* from denatured serum globulin in the presence of certain haptens. The claimed "antibody" formed a precipitate with haptenic agent.

There is no doubt that such an observation with great potentialities has been the object of many unsuccessful experiments in various laboratories, but only few have published their findings. In similar experiments, Haurowitz *et al.* (1946) found that the precipitates obtained in such experiments are not due to the effect of antibodies but are brought about by the non-specific flocculation of globulins charged positively at pH 5.0 to 5.5 by negatively charged azoproteins. Kuzin and Nervaeva (1947) could not detect the formation *in vitro*, using the method of Pauling and Campbell, of antibody to polysaccharides from Type III pneumococcus, gum arabic, *Shigella dysenteriae*, *S. paradyenteriae* Flexner, and *Streptococcus hemolyticus*. Also, no antibodies were observed in solutions against dye haptens. Similarly, no antitoxin formation was observed during the dehydration of serum proteins in the presence of the toxin from *Corynebacterium diphtheriae*.

In an earlier section of this work, we arrived at the conclusion that



all antigens fulfill the requirements of the criteria of ideal catalysis, they must, therefore, be considered as catalysts. Since all proteins are antigenic, they exhibit, therefore, biocatalytic activities. In Northrop's postulates, our concept of antigens acting as catalysts has undergone a variation by which antigens have been considered as acting as coenzymes. This variation contains certain concepts of the mechanism of antibody formation fundamentally different from ours. His antigenic coenzyme is supposed to be capable of initiating autocatalytic conversion of normal to antibody globulin. In contrast, our concept deals with a mechanism of the development of the specificity of antibody globulin structure preceding the completion of the synthesis of a normal globulin molecule, under the directive influence of the active units of the antigenic molecule. In other words, *the specificity of an antibody molecule is the consequence of specific cellular synthethic processes catalytically modified by an antigen to conform with the configuration of certain active groups of the antigenic molecule.*

The use of the term *coenzyme* to account for the postulated role of antigen in connection with the formation of specific antibodies introduces confusion. A comparison, therefore, of the role of coenzymes in respiratory processes, and the role of antigens in the formation of antibodies seems to be required. Haptens, which constitute the prosthetic groups of conjugated antigens, are incapable of inciting the formation of specific antibodies without combination with a protein. This would indicate that the catalytic role of an antigen resides in the protein molecule and that only in this combination can a hapten exercise its determinant function. The coenzyme groups of the respiratory enzymes are likewise inactive when not in combination with specific proteins. The same coenzyme group seems to function exclusively with certain specific proteins, and not with all proteins, yielding a group of related enzymes, for example, flavoproteins, each characterized by distinctive specificity of action. This specificity resides in the protein molecule and not in the coenzyme group. On the other hand, in immune reactions if the conditions permit, the same hapten can be combined with any antigenic protein to demonstrate its determinant characters. In this respect, a hapten is not selective. In these combinations, both the protein and the hapten produce antibodies, respectively specific.

In conjugated enzymes, neither the specific protein nor the coenzyme



group are by themselves active; they are interdependent. In conjugated antigens, while the determinant character of the hapten is dependent on its combination with a protein, the antigenicity of the latter is independent of the haptenic group. These basic differences in the relationship of the coenzyme group to the specific protein in conjugated enzymes on the one hand, and that of a determinant prosthetic group to the protein component of a conjugated antigen on the other, makes the assumption that antigens function as coenzymes endowed with autocatalytic powers unwieldy. Furthermore, the results of studies with bacteria and other cells fail to show that a coenzyme group is capable, adaptively or otherwise, of initiating the synthesis of a new protein in a cell specifically reactive with the particular coenzyme where there is none to be found to start with. In contrast, an antibody is produced *in vivo* only when there is an antigen present. This contrast likewise shows the lack of an experimental basis for an analogy between a coenzyme and an antigen.

f. **Specificity of Cleavage Products Derived from Antibody.** How far down in molecular size an antibody molecule can be brought proteolytically, or otherwise, before it loses every vestige of reactivity with homologous antigen is a pertinent fundamental question which has not as yet been adequately investigated. An antigenic protein hydrolytically split into polypeptide units would lose the ability of stimulating antibody formation, but would maintain the ability to combine with antibody and thus inhibit its reaction with whole antigen. The antibody combining abilities of smaller molecular entities are therefore inherent in these as well as whole molecules from which they are derived. Since these non-antigenic haptens are serologically specific, they must differ in this respect from the corresponding entities present in other proteins derived from the same species. It is reasonable therefore, to assume that there must function enzyme reactions specific for the synthesis of each protein and also for the synthesis of its haptenic parts which are obtainable by the hydrolytic cleavage of the whole molecule.

In a consideration of the structural difference between  $\gamma$ -globulin and antibody globulin (page 139), it was pointed out that diphtheria antitoxin (mol. wt., 184,000) on digestion with proteolytic enzyme gave a crystalline product (mol. wt., 90,000) 90 per cent precipitable with diphtheria toxin (Northrop, 1942). Immunologically, this product,

possessing  $\frac{1}{2}$  the size of the original antitoxin molecule, was distinct from the normal serum proteins. It would thus seem that the antitoxin freed from the normal serum components behaved as a new species of protein molecule. This is understandable if we accept the interpretation that antigens function as catalysts, similar in this respect to other specific enzymes involved in protein syntheses.

Related to the above finding, are the results of a study by Weil, Parfentjev and Bowman (1938). They reported that antibody globulin can be subjected to proteolytic digestion in a manner whereby it almost completely loses its ability to incite the formation of specific antibodies, without suffering loss in antitoxic properties. Diphtheria antitoxic globulin after partial peptic digestion at pH 4 and 4.5 was from 70 to 80 per cent non-coagulable by heat. The antitoxic component of the digest was separated by ammonium sulfate fractionation and purified by dialysis. The traces of remaining pepsin were eliminated by a freshly prepared suspension of calcium phosphate. This preparation was compared, in various serological and animal tests, with normal and immune horse plasma, and antitoxin globulin salted out with ammonium sulfate. Absorption experiments demonstrated directly that the antitoxic property was in the hydrolytically cleaved part of the solution and not in the unchanged fraction. The results of experiments showed that unimpaired—and even increased—antibody-function was possessed by this derivative of the antibody molecule. This derivative was so far removed that its property to function itself as an antigen was eliminated, and with it all traces of its original connection, detectable by immunological methods. The above results show that an antibody molecule can be stepwise split into various derivatives. In the first example, its serological relationship to normal serum proteins was eliminated without a loss in antigenic and antitoxic properties. In the second example, its antigenic relationship was eliminated without a loss of antitoxic activity. These facts show strongly that the groups responsible for the properties of antitoxin to combine with and neutralize toxin are distinct molecular entities derivable from the larger whole molecules.

In a similar study, Peterman (1946) reported that the early effect of papain or bromelain on horse diphtheria antitoxin and beef serum globulin is quite similar to that of pepsin and trypsin. The molecules are split into halves. On prolonged digestion, these halves are split into

quarters. The observed molecular weights of the quarters were 47,000, though, on the basis of the molecular weight of 153,000 of the original globulin particle, they should have been only 38,000. Although 90 per cent of the globulin was split into quarters, much antibody activity was retained. The antitoxin activity of the whole digest was from 70 to 90 per cent of the undigested antitoxin when papain was used, and 100 per cent of the activity was retained when digestion was carried out with bromelin. In another study, Peterman (1946) found that human immune gamma globulin is split by pepsin into molecules of half size. Further digestion yielded smaller particles and ultimately dialyzable fragments. Immunological assays showed that in the digests containing half size antibodies the typhoid "O" agglutinin was lost, while the concentration of "H" agglutinin, of diphtheria and streptococcus antitoxin and of anti-influenza A remained substantially unchanged.

In view of the above findings, one may expect that the specificity of antibody molecules may reside in still smaller units derivable from the quarter size digestion products. Dialyzable units of antibody may fail to agglutinate bacteria, or precipitate antigens, but they may inhibit the reactions between whole antigen and whole antibody.

Our information concerning the structure of proteins may be expanded if the smaller proteolytic or hydrolytic cleavage products are isolated and characterized chemically and serologically. One may be able profitably to employ the technique developed by Tiselius (1947) and Martin and Synge (1945) for this purpose. The results of such studies may have specific bearing on the debate on the valency of antibody molecules, and on the mechanism of antibody synthesis.

**Conclusion.** A consideration of the above experimental facts shows clearly that biological specificities are inherent in the respective chemical structures of proteins. The discussed material can be summarized in the following manner:

(a) The enzymes possessing minimum unit molecular weights of about 15,000, that is the smallest molecular weight entities which cannot reversibly be split or dissociated, are serologically and enzymatically distinct and different from the other proteins related to them species specifically.

(b) Polypeptides with molecular weights of about 6,000 show

specific combining activities with enzymes, and haptenic qualities. Hydrolytic cleavage products which are dialyzable, having molecular weights ranging from 600 to 1,000, exercise serologically specific activities demonstrated by the whole original molecules from which they are derived.

(c) Antibodies of  $\frac{1}{4}$  to  $\frac{1}{8}$  size of the original molecule show activities comparable to original activities. Antibodies which proteolytically can be reduced to  $\frac{1}{2}$  size with a loss of their species relationship to normal proteins, and antibodies which proteolytically can be changed to such forms that represent loss of their antigenic qualities without loss of their antitoxic activities, show clearly that these biological specificities are inherent in their respective chemical structures. It can therefore be inferred that these specificities arise from specific synthetic enzyme reactions. The summation of the specificities of the smaller structural units represent the various specificities of the whole protein molecule. The concept of the formation of various proteins from proteinogen, as discussed at the beginning of this section, does not appear to be capable of accounting for the above enumerated specificities of structural units. In other words, our view is that *the specificity of an antibody molecule is the consequence of specific cellular synthetic processes catalytically modified by an antigen to conform with the configuration of certain active groups of the antigenic molecule.*

The change a certain specific protein undergoes from its inactive to its active state, for example, trypsinogen to trypsin, whether by a shift in the pH of the surrounding medium, by an ion effect, by non-specific agents such as concentrated solutions of ammonium or magnesium sulfate, or by the presence of an enzyme to accelerate the spontaneously occurring change, in an autocatalytic manner or otherwise, appear to be a superficial chemical change, and do not involve deep-seated changes to merit the assumption that a new protein is derived from another protein. The enzyme precursor idea does not appear satisfactory as the basis for such fundamental questions pertaining to the synthesis of enzymes, antibodies, viruses and the like. The results obtained from immunological and chemical studies appear to contradict the postulate that one protein is autocatalytically formed from another protein, or from a "master proteinogen."



## 8. Anti-Antibodies

According to the theories of Breinl and Haurowitz, Mudd, Pauling, and Alexander, etc., the antigens modify the configuration of the normal globulins during their synthesis, resulting in the formation of specific antibody globulins. The antigens exercise this influence to this extent and apparently not further. The statements by Breinl and Haurowitz to the effect that "*the orientation of amino acids in the formation of antibodies . . .*"; and the "*coupling of amino acids to the peptide occurs in an orienting environment, namely, the antigen-protoplasm interface.*" (Mudd), need not therefore be interpreted to imply that amino acids in an antibody molecule occupy different positions in the peptide chain than those of normal globulins. It may simply mean, as stated by Mudd, that "*the chemical groupings coupled to the molecule undergoing synthesis at the antigen surface must be adapted spatially.*"

The studies and conclusions of Landsteiner and van der Scheer (1928) were instrumental in the formulation of the above concept. They demonstrated that the steric configuration of antigens exercise distinctive influence on the serological specificity of the antibodies against them; l- and d-antigenic isomers react specifically with the respective antibodies. These findings brought definite proof for the view that the steric configuration of the antigenic groups is one of the factors determining serological specificity. The mere difference in the position of H, OH and COOH in l- and d-antigenic isomers sufficed to alter the specificity of the antibody. They also suggested that the steric configuration may play a significant part in the serological specificity of carbohydrates such as those discovered in bacterial antigens. This view has been amply confirmed by the extensive studies of Avery and Goebel, *et al.* Since this subject will be discussed in a latter part of this study, it suffices here to mention that the antibody against galacto-albumin reacted with solutions of galacto-globulin and galacto-albumin. These antibodies, however, failed to precipitate glucoside albumin. The antibody produced against the antigen containing  $\alpha$ -glucoside reacted selectively with this antigen, and not with the antigen containing a  $\beta$ -glucoside group, and *vice versa*. A difference in the spatial arrangement of the same polar group therefore exercised



definitive influence on the serological specificity of the antibodies produced.

The optical antipodes, as well as space isomers differ chemically and physicochemically, exercising different degrees of attractive and repulsive forces. Atoms or atomic groups falling within the influence of these forces are either repulsed out of the sphere of influence or are attracted to combine. The optical and space isomers thus manifest different properties in combining with proteins, functioning as a substrate for an enzyme, in bacteriostatic action, in enzyme inhibiting effect, in forming racemates, etc. During the synthesis of globulin molecules under the influence of antigens containing polar groups, or optical and space isomers, such influences do, no doubt, come into play. It could thus be assumed that during the synthesis of the globulin molecules the atomic groups rotate their position in space in accordance with the configuration of the antigenic molecule, resulting in the formation of antibody as modified globulin.

**a. Presence of Serologically Reactive Basic Amino Groups in Antibody Globulins.** Mudd and Joffe (1933) found that when antisera were treated with formaldehyde before combination with antigen, agglutination was consistently inhibited. The antibodies treated with formaldehyde showed decreased basicity, which was evidenced by a shift in the isoelectric point of the sensitizing film toward the acid side by about 0.6 to 0.8 pH unit, and reduced agglutinating tendency of the sensitizing film. Chow and Goebel (1935) investigated chemically and serologically the function of the basic amino groups in Type I anti-pneumococcus precipitin. Eighty-eight per cent of the purified antibody they studied was precipitable by Type I specific carbohydrate. The remaining 12 per cent protein was considered as representing antibodies against cellular proteins of the pneumococcus and for the somatic carbohydrate present in the original serum. The isoelectric point of the precipitin was unusually alkaline, i.e., pH 7.6. This was attributed to the presence of either a relatively high ratio of amino to carboxyl groups or to the presence of a higher percentage of basic amino acids in the antibody molecule than the globulin of normal serum would contain. The distribution of basic amino acids in the globulin of normal and immune horse serum, aside from a slightly higher lysine content of the latter, showed no essential difference. Acetylation of antibody revealed the presence of one acetyl group for

every primary amino group in the original antibody molecule. Acetylated antibody lost to a great extent its capacity to precipitate the type specific polysaccharide. When relatively high concentrations of type specific carbohydrate were added to the acetylated antibody, a precipitate was formed. The original antibody gave +++ precipitate with 1:1,024,000 carbohydrate dilution; in contrast, acetylated antibody gave +++ with 1:4000 and + precipitate with 1:8000 carbohydrate dilution. These results were interpreted to indicate, besides the amino groups, the presence of certain other reactive groups likewise involved in the precipitation reaction.

Since formalized antibody protein gave no precipitation test the amino groups in the native antibody were believed to be concerned in the union of the antibody with the carbohydrate. Deformalization of the antibody restored its serological specificity. The loss of serological specificity of the formalized antibody was considered presumably due to the conversion of the  $-\text{NH}_2$  to  $-\text{N}=\text{CH}_2$  group. Conversely the esterification of the  $-\text{COOH}$  group of the antigen carbohydrate deprived it of its reactivity with the native antibody. Saponification of the esterified carbohydrate fully restored its reactivity. Though the saponified carbohydrate still contained methyl groups (as a result of the treatment of the carbohydrate with diazo-methane in the esterification experiment) bound both on the primary  $-\text{NH}_2$  group of the parent carbohydrate and as a methoxyl group attached probably to one of the hydroxyl groups, unlike the inert ester, it reacted readily with antisera. These results showed that a free  $-\text{COOH}$  group in the carbohydrate was of primary importance in rendering it serologically reactive. In other words, the union of the free  $-\text{COOH}$  group in carbohydrate with a free  $-\text{NH}_2$  group in the antibody was considered possibly necessary for the serological reactions. The normal globulin molecule does not possess this serologically reactive "free amino group."

The presence of a serologically reactive "free amino" group in antibody globulin and not in normal globulin was also shown by Goebel and Hotchkiss (1937) with substances containing organic acid radicals. They found that artificial azoprotein antigens containing organic acid radicals, quite unrelated in chemical constitution to glucuronide or galacturonide, precipitated in antipneumococcal horse sera. Antigens prepared from *p*-aminobenzene carboxylic and sulfonic acids, though not reactive in normal horse serum, precipitated in antipneumococcus

sera. The precipitation of these antigens in Type III immune horse sera was inhibited *indiscriminately* by the sodium salt of any one of the uncombined acidic derivatives (*p*-aminobenzene sulfonic acid, *p*-aminobenzene carboxylic acid, *p*-aminobenzylglucuronide, *p*-aminobenzylgalacturonide as inhibiting substances against the test antigens prepared from them and chick serum).

The above cited substances containing  $-\text{COOH}$  and  $-\text{SO}_3\text{H}$  groups, possess but one property in common, namely, acidic groups of divergent nature. As stated by Goebel and Hotchkiss the reaction of these antigens in antipneumococcal horse sera represents a neutralization of the charge of basic groups of the antibody protein by the acid groups of the non-specific haptens, followed by precipitation.

**b. The Directive Influence of Optically Active Catalysts in Producing Optically Active Substances.** The presence of serologically reactive basic amino groups in antibody globulin may appear to indicate that during its synthesis the position of the basic  $-\text{NH}_2$  groups had been specifically oriented by the presence of  $-\text{COOH}$  polar groups of the pneumococcal carbohydrate, or synthetic glucoproteins. In contrast, during the synthesis of normal globulin, the position of the amino groups not being directed by foreign influences, do not occupy any specific position with reference to foreign substances.

It can be visualized that, similar to the orienting influence of  $-\text{COOH}$  groups in the pneumococcal carbohydrates, optically active groups in d- and l-antigens (Landsteiner and van der Scheer, 1928, 1929) may specifically orient certain groups during the synthesis of the antibody globulin molecule to conform with the optical configuration of the antigens. The question as to whether d- and l-antigens, acting as optically active catalysts, have produced optically active specific groups in the antibody molecule is of general interest; to this question we have at present, no direct answer. It has been observed by numerous investigators that in general, it is characteristic of living cells to metabolize or synthesize only one mirror image of optically active substances. This fact has been known since the time of Pasteur and the literature is rich in confirmatory evidence which we need not discuss here.

In relation to the question raised above whether optically active antigens produce antibodies with specifically oriented optically active groups responsible for the serological reactivity, it might likewise

be inquired as to whether the cellular enzymes which catalyze one-sided asymmetric synthesis or metabolism (i.e., either l- or d-isomer) of optically active substances are themselves optically active.\* It has been claimed (Helferich, *et al.*, 1938) that  $\beta$ -d-glucosidase and  $\beta$ -d-fructosidase contain carbohydrate-like substances of glucose nature; and it is postulated that these enzymes by virtue of the carbohydrate groups, perhaps with  $\beta$ -configuration, combine with and catalyze mirror image substrates. The active group of the enzyme is assumed to form a racemate with the substrate in accordance with the "Lock and Key" idea of Emil Fischer (Emil Fischer, 1894, 1898; see also Lettré, 1937). These claims and postulates though of utmost theoretical interest are not as yet experimentally demonstrated.

On the other hand, it has been definitely shown that optically active d- and l-active organic catalysts orient specifically the configuration of the atomic groups on asymmetric carbon atoms produced during the synthesis of optically active substances from optically inactive starting materials. By an accurate consideration of these laboratory processes, which take place continuously in the living cell, we might be able to understand at least the principles involved in asymmetric synthesis.†

Rosenthaler (1908, 1909, 1913) obtained the first proof that an enzyme in emulsin which he presumed to be optically active could

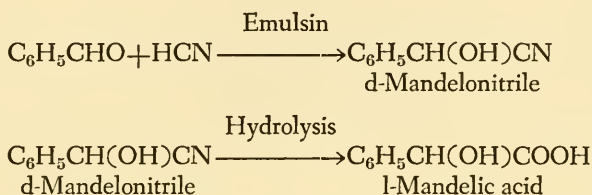
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\*Pasteur suggested that every asymmetry owes its existence to some asymmetric forces operating at the moment at which the asymmetry appeared. Emil Fischer (1894, 1898) stated that one active molecule gives rise to another, and the formation of sugar in plants takes place by chlorophyll which he assumed to be optically active. The optical activity of chlorophyll has recently been demonstrated by Stoll and Wiedemann (1933).

†In evaluating the reports concerning the optical purity of an enzymic system or asymmetric synthesis with a preponderance of one of the antipodes, the following considerations must be kept in mind. From the standpoint of true catalysis there is no difference in the energy required for the interconversion or the formation of the l- and d-antipode of a given substance in equal concentrations. Analyzing the principles of the asymmetric synthetic action of enzymes Werner Kuhn (1936) stated that from the thermodynamic point of view the optically active state is not a state of equilibrium as compared with the racemic state. The mixing of the two antipodes to form a racemate liberates energy, while their separation requires an expenditure of energy. According to Kuhn, in certain instances, the preponderance of optical purity in a system, despite the gradual decrease of optical activity in a single enzymatic process, can be explained by "stereo-autonomic" behavior of some substances. That is, this behavior conditions stable optical purity of the synthetic product. It is known that d-mandelonitrile, synthesized by the plant, is stabilized in the form of the less soluble and easily crystallizable  $\beta$ -gentiobioside (natural amygdalin). In contrast the more soluble gentiobioside of l-mandelonitrile remains in solution. The excess l-mandelo-



influence the combination of benzaldehyde and hydrogen cyanide so that the reaction took place one-sidedly, the mandelonitrile formed being dextrorotatory. The extent of the asymmetric synthesis must have been considerable for the *dextrorotatory* nitrile was formed in large excess over its antipode since l-mandelic acid obtained on hydrolysis of the nitrile was optically pure after two crystallizations from benzene.



This was the first definite example of an asymmetric synthesis carried out in the laboratory by an enzyme. Rosenthaler (1913) found in the leaves of *Taraktogenos Bluenci* HSSK also an enzyme, called *l-oxynitrilase*, which brought about the formation of l-mandelonitrile from benzaldehyde and hydrogen cyanide, as distinct from the common d-oxynitrilase which forms d-nitrile. The emulsin from cherries was found by Krieble (1913) to yield sometimes a d- and sometimes an l-nitrile.

Working with bacteria as an enzyme source Mayer (1926) was able to obtain from phenylglyoxylic acid and hydrocyanic acid l-mandelic acid by *Bacterium ascendens*, and d-mandelic acid by lactic acid bacteria.

Bredig and Fiske (1912) made similar observations using optically active organic catalysts. They dissolved 50 ml. of benzaldehyde (0.5 mole) in 170 ml. of chloroform (as solvent) and treated the solution with anhydrous hydrocyanic gas (0.5 mole). After one hour at 25°C.,

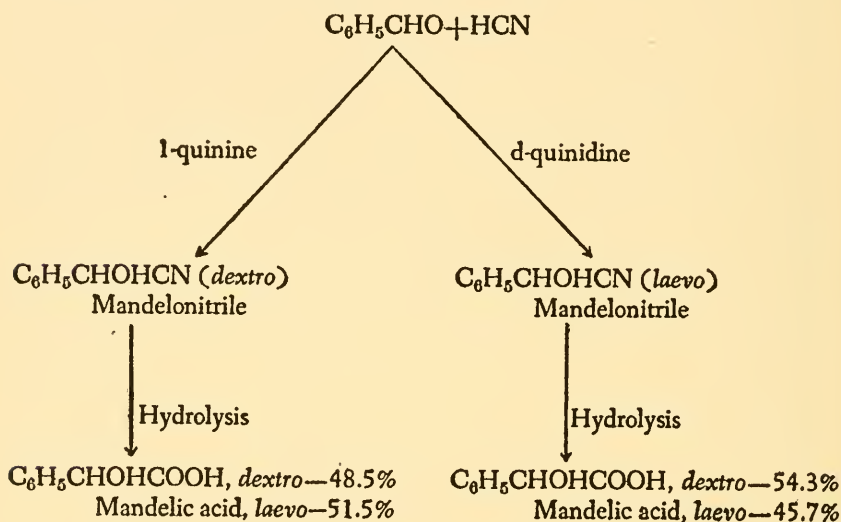
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nitrile can thereupon be racemized according to the requirements of true catalysis; that is, the d-nitrile originating from excess free l-nitrile is again transformed into  $\beta$ -gentiobioside and the process continues until all the l-nitrile is converted into the less soluble glucoside or d-mandelonitrile.

The preponderance of optical purity in an enzymatic synthetic process is observed also: (a) when widely different velocities in the formation of the two optical isomers determine the degree of preponderance of the d- or l-isomer in the substance synthesized; (b) an increase of optical purity can be observed when the reaction is interrupted before the optical activity disappears as the result of gradual racemization (Langenbeck and Triem, 1936); (c) by the elimination from the living system without utilization (Abderhalden and Samuely, 1906), or by more rapid metabolism of the unnatural optical isomer (Krebs, 1933).



it was treated with 0.5 g. (0.0015M) quinine or quinidine alkaloid as catalysts. After 24 hours the reaction mixture was treated with 100 ml. of 4 N aqueous sulfuric acid and shaken for five minutes to remove the catalyst alkaloid. A study of the chloroform solution showed that the alkaloid had acted as a specific directive catalyst in the formation of nitrile. *Laevorotatory* quinine caused the formation preponderantly of a *dextrorotatory* nitrile, from which *laevo*- and *dextrorotatory* mandelic acids were obtained on hydrolysis. In contrast, *dextrorotatory* quinidine gave preponderantly *laevorotatory* nitrile:



Analogous results were obtained when cinnamic aldehyde, anisaldehyde, citral, piperonal and acetaldehyde were used in the place of benzaldehyde.

Bredig and Fiske believed that alkaloids and cyanhydrin form a complex compound as a necessary condition for catalytic synthesis. They found that the alkaloids disappeared from the aqueous solution into the chloroform or toluene solution of cyanhydrin; only cyanhydrins appeared to participate in such a combination. McKenzie (1936), commenting on the findings of Bredig and Fiske, cited Isobel A. Smith's interpretation of the findings of Traube and Onodera (1914) who found that alkaloids of high molecular weight, such as atropine or quinine, formed colloidal solutions in water, while their salts formed true solutions. Accordingly it appeared to Smith that

herein in all probability lay the asymmetric synthesis; in the chloroform solution these alkaloids formed colloidal adsorption compounds acting in a capacity very similar to that of an enzyme.

In this connection it may be mentioned that Bredig and Gerstner (1932) made the interesting observation that by introducing the diethylamino group into cotton fibre the latter was rendered catalytically active in effecting the synthesis of l-mandelonitrile from benzaldehyde and hydrogen cyanide.

Rosenthaler (1909) extending the scope of his observations later obtained also l-, d-, and i- (inactive) forms of nitrile from numerous aldehydes and hydrocyanic acid. Acetaldehyde, isobutyraldehyde, heptaldehyde, furfural, o-methoxybenzaldehyde, anisaldehyde, cinnamic aldehyde, etc. yielded d-nitriles; only citral and o-phthalaldehyde yielded l-nitrile; chloral, salicylaldehyde, m- and p-oxybenzaldehyde, p-nitrobenzaldehyde, methyl-ethylketone yielded optically inactive nitriles. These results were interpreted to indicate that either the emulsin preparation contained more than one optically active enzyme, or there exists a certain type of substrate specificity in the synthesis of stereochemical isomers. The results obtained by Bredig and Minaeff (1932) with organic optically active catalysts in some respects were analogous to the above observations made by Rosenthaler (1909).

TABLE III  
(Bredig and Minaeff)

Starting material	Catalysts		Synthetic product
	d-Quinidine	l-Quinine	
Cinnamic Aldehyde	present	----	l-rotatory
Cinnamic Aldehyde	----	present	d-rotatory
Anisaldehyde	present	----	l-rotatory
Citral	----	present	d-rotatory
Piperonal	----	present	d-rotatory
Acetaldehyde	----	present	l-rotatory

The above results show, as Rosenthaler found, that the specificity of substrate may likewise play a role in defining the optical configuration of the final reaction product.

Granting that d- and l-active antigens might catalyze the synthesis of homologous antibodies, comparable to the catalytic synthesis of d- and l-active substances respectively by l- and d-active organic catalysts, it would then appear reasonable to expect that hypothetically d-antigen catalyzes the synthesis of l-antibody and l-antigen that of d-antibody. The serological reaction between the optically active antigens and the homologous antibodies would then be comparable with the formation of a racemate such as d-cinchonine-l-tartrate. This racemate is a relatively more insoluble molecular combination than either of the components, which fact might be compared with the formation of an insoluble precipitate by the reaction between the antigen and antibody.

The protein molecule is made of optically active amino acids which have the l-configuration. Lettré (1937) postulated that in the antibody molecule the serologically determinant group as the prosthetic group is the mirror image of the naturally occurring l-active groups of the normal globulin molecule. A simple protein acting as antigen would produce accordingly an antibody containing as many d-active prosthetic groups as there are antigenic l-active groups in the simple protein. Though this concept might appear to account for the formation of hypothetical d-active antibodies against l-active antigens, difficulty arises when we attempt to explain the nature of the active groups in the antibody molecule produced against d-antigens. Since the antibody against the latter would hypothetically be expected to have the l-configuration, it is difficult to conceive that there can be two kinds of l-active globulins to account on one hand for the serological reactivity of the antibody against the d-antigen, and a second one corresponding to normal globulin made of l-active amino acids on the other.

*Antibody Carbohydrate as a Possible Seat of Specificity.* Perhaps it is pertinent to the question discussed above to consider the possible rôle of the carbohydrate contained in the antibody globulin on the specificity of serological reactions. As far as we know, this question has never been raised or studied before. It has been shown by Hewitt (1938) that normal serum globulins contain chemically bound carbohydrate groups. Working with normal serum fractions which were distinguishable by biological, chemical, and physical methods he found that seroglycoid, globoglycoid and pseudoglobulin, respectively,

contained 5.6, 6.2 and 2.2 per cent galactosemannose and 2.7, 2.9 and 1.1 per cent glucosamine. Euglobulin, pseudoglobulin, globoglycoid and seroglycoid, respectively, contained 4.1, 3.3, 9.3, and 8.4 per cent polysaccharide.

Remington and Van der Ende (1940) reviewed the problem by preparing crystalline albumin, crystalline globoglycoid, and seroglycoid and seromucoid from horse serum. According to them seromucoid contained 10.7 per cent of a polysaccharide consisting of N-acetyl-d-glucosamine, d-mannose and d-galactose in equimolecular proportions. Crystalline albumin and globoglycoid are essentially identical and differ from seroglycoid. The latter, although closely related to seromucoid, was not identical with its immunological properties.

The purified antitoxic pseudoglobulin contained 2.6 per cent of carbohydrate calculated as mannose-galactose-glucosamine. Peterman and Pappenheimer (1941) showed that after digesting diphtheria antitoxic material with pepsin the carbohydrate moiety remaining with the antitoxic portion of the molecule amounted to 3.8 per cent. Northrop (1942) working with crystalline diphtheria antitoxin showed the presence of not less than 2 per cent carbohydrate as part of the molecule.

Immune  $\gamma$ -globulins from human, horse and bovine sources were analyzed by Smith, Green and Bartner (1946) and were found to contain from 1.26 to 1.5 per cent hexamine. The ratio of hexamine to hexose was 1 to 2.

During the synthesis of antibody globulin resulting in the orientation of various groups in accordance with the configuration of the antigenic molecule one could expect that the polar groups in the carbohydrate moiety of the molecule may likewise undergo similar orientation. The specificity of the antibody with reference to the optical configuration of the homologous antigen might in particular be a property of the carbohydrate group of the antibody molecule. In this connection the important question is whether or not d- and l-active, or  $\alpha$ - and  $\beta$ -antigens produce, respectively, l- and d-active groups, or groups with  $\alpha$ - and  $\beta$ -configurations in the antibody carbohydrate group, in a manner comparable to the action of d- and l-active organic catalysts discussed above. At present we have no answer to any one of these questions.

**c. Experiments Dealing Directly with the Question of Anti-Antibody Formation.** If the synthesized antibody molecule possesses

a stereochemical correspondence with the antigen, as appears to be the case from various studies, for the reason that its spatial configuration has been oriented to fit the spatial configuration and the affinities of the polar groups and of antigenic optical antipodes and space isomers, it would appear reasonable to expect that the differences between antibodies against l- and d-antigens are sufficiently great to render them antigenically distinguishable. At least it would seem that these antibodies should spatially be sufficiently different to make them antigenically different from the normal globulins.

Pauling's concept of the antibody molecule that "only in the configuration of the chain, that is, in the way that the chain is coiled in the molecule," could not, in our opinion, be viewed simply as a physical change not involving a change in the spatial position of the atoms or groups. For it is stated by Pauling that the atoms and groups which form the surface of the antigen will attract certain complementary parts (positively and negatively charged groups). This may imply that various groups in the globulin molecule may undergo spatial rearrangement as a consequence of the said polar property of groups to yield a new configuration in the coiled chain end of the molecules.

It would therefore appear that if a difference in the position of H and OH in R- $\alpha$ -glucoside or R- $\alpha$ -galactoside antigens and those in d- and l-tartranilic acid antigens are capable of producing specific antibodies, it is reasonable to ask why possible changes in the spatial arrangement of the polar groups, NH<sub>2</sub>, COOH, OH, CONH, SH, largely responsible for the chemical reactivity of the proteins, should not be considered also as possible factors in the formation of anti-antibodies against these antibodies.

To obtain an answer to the question whether or not anti-antibodies could be produced numerous investigations have been carried out since the time of Ehrlich. The results, however, have not been of a definitive nature to effect a clear cut differentiation between the antigenicity of normal and antibody globulins derived from a given species of animal. The reason for failure to do so apparently is to be found, as recent studies have shown, in the fact that the antibody globulin molecule is composed in part of an inactive portion which is indistinguishable from normal globulin. The only clear proof that diphtheria antitoxic activity may be exhibited by a protein which is antigenically distinct from normal globulin is provided by Northrop



(1942) who isolated the antitoxin in crystalline form after enzymic digestion of the original complex antitoxic globulin and by eliminating the inactive component.

Despite the undecisive nature of the results of the investigations carried out previous to that of Northrop it might not be in vain to present briefly the nature and the type of these experiments. It is to be noted that because of its theoretical importance this question has been an object of study by older immunologists, namely, Bordet, Ehrlich, Sachs, Morgenroth, Pfeiffer, Friedberger, etc., and immunologists of our times, Landsteiner, Heidelberger, Marrack, and others and their collaborators.

Ehrlich and Morgenroth (1901) carried out cross immunization experiments with ox, goat and rabbit blood cells, and then, with the adsorption technique, they arrived at the conclusion that plural immune bodies are produced by injections of ox and goat blood. By effecting differentiation of various groups of immune bodies by means of the anti-immune bodies they believed they had shown the formation of anti-antibodies.

Bordet (1899) demonstrated the formation of anti-hemagglutinins (anti-amboceptors). Fowl's serum injected intraperitoneally into a rabbit produced an anti-immune body which protected rabbit red blood corpuscles against the hemolytic action of fowl's serum.

In a subsequent extensive study Bordet (1904) observed that red blood corpuscles of different species of animals, sensitized by appropriate hemolytic sera obtained from a species B, lose their sensitivity to complement when treated with the anti-amboceptor. In certain instances it was not necessary to use for immunization the serum containing specific amboceptor; it was sufficient to inject the animal, namely the guinea pig, with normal serum of species B, which Bordet stated, contains normal amboceptors. (See also Muir and Browning, 1906).

Friedberger and Moreschi (1908) stated that in their earlier experiments they believed they had demonstrated the formation of anti-amboceptors by immunizing goats with hemolytic immune rabbit serum against goat red corpuscles. In later experiments, contrary to their expectations, when the serum of a rabbit immunized with goat's serum hemolytic for rabbit corpuscles was added to rabbit red corpuscles sensitized with goat amboceptor the hemolysis in the presence of

complement was not prevented. On the contrary, they observed an acceleration of hemolysis.

In a subsequent study Moreschi (1908) observed that red blood corpuscles, which were sensitized with a non-agglutinating dose of a corresponding immune serum and washed free of excess serum, treated with a small amount of a precipitin containing serum underwent readily a strong agglutination. He also observed the same effect in bacterial agglutinations. Altman (1912) using sensitized and washed red blood cells as immunizing agents obtained results similar to those of Friedberger and Moreschi (1908).

The formation of anti-antibodies capable of neutralizing the lytic action of antibacterial immune sera were reported by Pfeiffer and Friedberger (1903). It was, however, immaterial whether the rabbits were immunized with normal or artificially immunized goat serum. Pfeiffer and Friedberger interpreting their results were inclined to assume that various antibodies supplied by the same species of animal, speaking in Ehrlichian terms, possess a common group, which characterized the species of animal from which it originates, and that the anti-immune-serum in some way must be related to this group.

Dehne and Hamburger (1904) had shown that normal horse serum precipitinogen as a normal constituent of horse serum is closely associated with the horse antitoxins. Kraus and Pribram (1905) reported that horse sera containing a high agglutinin titer for typhoid bacilli were completely absorbed out by anti-horse rabbit serum. Experiments with anti-cholera immune horse serum yielded similar results. The anti-bacterial horse antibodies and normal horse serum precipitinogens were shown to possess a common combining group.

Landsteiner and Prasek (1911) immunized rabbits against goat serum. The immune serum reacted strongly with goat serum. The immune rabbit serum inhibited goat agglutinins, particularly the bacterial agglutinins. These facts indicated that precipitins acted as anti-agglutinins.

**d. Non-Identity of the Combining Sites for Antigen and Antibody in an Antitoxin Molecule.** Eisler (1920) found that horse serum antitoxin (tetanus, diphtheria) could be precipitated by a rabbit immune serum against horse serum even after combination with toxin. Smith and Marrack (1930) showed that antitoxin and toxin-antitoxin flocules react like pseudoglobulin with anti-pseudoglobulin serum. They

stated that since antitoxin, when precipitated by a precipitin, can still combine with toxin, different groups must be involved in the two reactions. Eagle (1936) stated, as previous investigators had shown, that a molecule of antibody can function either as an antibody or as an antigen. In the former capacity, it combines with homologous antigen; in the latter capacity the antibody molecule is itself precipitated by an antibody to serum protein. Experimental details of Eagle's studies differ from those of Eisler, Smith and Marrack in that the latter did not saturate antitoxin with toxin or precipitin prior to the addition of precipitin or toxin. In Eagle's experiments horse antitoxin precipitated by a large excess of a rabbit antiserum against horse serum and presumably saturated with precipitin, nevertheless retained almost its original neutralizing activity. Antitoxin so completely saturated with toxin as to form a highly toxic compound combined with a rabbit precipitin against horse serum. From these and numerous other serological data, it is to be seen that antibody globulin molecules possess specific reactive groups not present in the normal globulin molecules.

**e. Further Experimental Data Concerning the Presence of a Common Group in Different Antibodies from a Species of Animal.** Ando and his associates (1937, 1938) immunized rabbits, ponies and horses with diphtheria toxin-antitoxin flocculi, and with the specific precipitates of pneumococcal type specific carbohydrate and its antibody in its purest form. The serological tests were carried out by the optimal-proportions-method of Dean and Webb and the absorption technique. They found that the precipitin-versus-antitoxin absorbed diphtheria antitoxin in the range of optimal proportions and also at higher ratios. Pneumococcal antibody, however, was absorbed by precipitin-versus-antitoxins only within the range of the mixtures containing far less amounts of anti-pneumococcal serum than that in the optimal proportion. Precipitin-versus-antibacterial antibody absorbed anti-pneumococcal antibody, but diphtheria antitoxin was absorbed by the same precipitin only in the secondary zone and not in the primary or main zone. These results were interpreted by the authors to signify that various antisera contain larger amounts of antibody against the homologous than the heterologous immunizing flocculi. In these studies as indicated above the horse antisera against different flocculi reacted with homologous as well as heterologous antisera.

Treffers and Heidelberger (1941) examined the properties of spe-

cific precipitates both as immunizing and as test antigens to determine quantitatively the chemical relationship, not only of antibodies to the different serological types of pneumococcus, but also of antibodies directed against a somatic component, the so-called C polysaccharide of the pneumococcal cell.

A specific precipitate derived from anti-pneumococcus Type II horse serum was used as antigen for the injections of rabbits and the resulting sera were tested against the specific precipitates obtained with:

- (a) Pneumococcus Type I, II and Group C carbohydrate and homologous horse sera;
- (b) H. Influenza Type B polysaccharide and anti-influenza Type B horse serum;
- (c) Diphtheria crude toxoid and horse diphtheria antitoxin;
- (d) Crystalline egg albumin and homologous horse antiserum; and
- (e) Specific precipitates from Type II anti-pneumococcal rabbit serum and from Type I anti-pneumococcal pig serum.

The results showed that "after absorption with egg albumin-anti-egg albumin (horse) specific precipitate and removal in this way of roughly one-half of the antibody the anti-specific precipitate rabbit serum still resembled the unabsorbed serum in its quantitative reactivity toward a high ratio pneumococcal specific precipitate when compared at the same antibody content.

"The co-existence of separate groupings which function as antigen and antibody on the same molecule of horse globulin is indicated by experiments showing pneumococcus Type II horse antibody precipitated with an excess of anti-specific precipitate rabbit serum can still combine with Type II specific carbohydrate, and that the horse antibody in a solution of the egg albumin-anti-egg albumin (horse) specific precipitate in an excess of egg albumin still reacts with the anti-specific rabbit serum."

Tests were carried out to examine whether the antibody specificity would affect in any way their function as test antigens. Neither by the sensitive quantitative agglutinin method nor by passive anaphylaxis tests in the guinea pig could any variation be demonstrated due to the specific antibody groupings. These and other findings led the authors to the conclusion that the antigenic reactions of this representative water-insoluble group of antibodies engendered in the horse are essentially similar. These findings confirm in a more rigorous quantita-



tive manner the conclusion of previous investigators that the various specific antibodies in a species of animal are closely related antigenically. Thus the only antigenic specificity demonstrable for the antibodies investigated was that due to their common origin, and "the groupings," as stated by the authors, "responsible for their antibody function, constitute either a small part of the total protein molecule or else are non-antigenic."

**f. A Comment on the Use of Antigen-Antibody Complex for the Production of Anti-Antibodies.** According to our present concept the determinant groups in antigenic molecules which specifically stimulate the production of antibodies *in vivo* are identical with the groups which combine with the antibodies produced. It would therefore be expected when these groups of antigens are saturated with antibodies the treated antigens would be deprived of their capacity to develop specific antibodies. Olitzki (1935) reasoning in this manner reviewed the previous studies concerning this question and found it controversial. He then set out to clarify this controversy.

He reported that the immunization of rabbits with *E. typhosa* sensitized with anti-serum to O-antigen did not reduce the production of H-agglutinin, but reduced the production of O-agglutinin to nearly 20 per cent of the normal immunization effect by an unsensitized vaccine. Sensitization of the vaccine by H-serum reduced only the production of H-agglutinin to 20 per cent of the normal immunization effect. Sensitization of the vaccine by both H- and O- serum reduced the production of both types of agglutinins. Olitzki found that the injection of sensitized antigen together with larger amounts of free antibody (immune serum) suppressed the formation of agglutinins completely. The effect was the same whether the excess serum was administered before or after the inoculation of the sensitized vaccine.

Phage lysed bacteria produced at least the same quantities of antibodies as whole bacteria. But after sensitization with the same quantity of immune serum, phage lysed bacteria were completely deprived of their ability to produce antibodies, while the phage untreated bacteria remained effective.

It is interesting to note in the results of Olitzki that after injection of the same quantity of free immune-agglutinins as used for sensitization the free agglutinins could be recovered in the serum. Especially the H-agglutinins were demonstrable in the serum on the next day



after the injection and persisted there during a period of ten days. However, the same amounts of antibodies injected after being fixed by the antigen do not appear in the serum.

It is evident from the above results that the combining groups of an antigen, when free, are able to produce *in vivo* antibodies with specific combining groups. When the combining or antigenic groups of a molecule are saturated with antibody and kept saturated *in vivo* with excess antibody they are unable to incite the formation of antibodies. Apparently the antigen + antibody  $\rightleftharpoons$  antigen-antibody complex equilibrium is completely shifted to the right in the presence of an excess amount of antibody.

The question may therefore be asked whether the reverse process does or does not take place. Namely, if the combining groups of an antibody are saturated with antigen the former may fail to incite the production of specific anti-antibodies, or antibodies to the specific configuration or combining groups of antibodies involved in the antigen-antibody reaction. The saturation of the combining groups of antibodies would not block the species specific antigenicity of the antibody globulins which would account for the formation of antibodies (when combined with antigen) which are reactive with normal globulins from the same species. In view of the above consideration one may question the validity of the conclusions drawn concerning the absence of anti-antibody formation in experiments involving the use of the above mentioned antigen-antibody complexes (Ando and associates, 1937, 1938; Treffers and Heidelberger, 1941) where combining groups of both reactants are mutually saturated, and therefore are incapable to negotiate the reactions required for the formation of anti-antibodies.

**g. Acquisition of New Antigenic Specificity by Antitoxins.** The question of whether or not antibody has acquired a new specificity antigenically distinct from the corresponding normal globulins has been investigated by various workers. Weil, Parfentjev and Bowman (1938) made the following observations: (a) antibody response in rabbits, immunized with pepsin-treated antitoxin, is slight and delayed; (b) tests for cutaneous hypersensitivity, and precipitin and complement fixation tests all showed that the treatment with pepsin had eliminated the original antigenicity of the antitoxin; and (c) guinea pigs sensitized to pepsin-treated antitoxin were shocked by normal horse serum, but not by the homologous antigen. The failure to shock the guinea pig

by pepsin-treated antitoxin has been suggested by other investigators to be due to the limited amount of antigen that could be injected. Coghill, *et al.* (1940) reported that diphtheria antitoxin which had been despeciated by treatment with taka-diastrase did not kill guinea pigs sensitized passively to normal horse serum, and only a few despeciated sera gave any anaphylactic reaction at all. The guinea pigs sensitized with serum treated with taka-diastrase could be shocked but only with large doses of the same antigen. Kass, Scherago and Weaver (1942) reported that ileum segments from guinea pig sensitized passively to normal horse pseudo-globulin failed to respond to sera digested with enzyme. Christensen and Kerrick (1948) confirmed this observation and found that guinea pigs sensitized to pepsin-treated serum will react when tested with the same antigen, but also show that this cannot be due to the remaining horse serum specificity, because pepsin-treated serum is significantly more effective in eliciting anaphylactic shock in guinea pigs sensitized to pepsin-treated serum than in guinea pigs sensitized to normal horse serum. They concluded that "pepsin-treated antitoxin not only has retained some of its original horse serum specificity, but in addition as a result of the process of purification, has acquired a new antigenic specificity." In this study there is not a detailed description of the purification of the pepsin-treated antitoxin, and, therefore, the presence of normal serum components is not excluded. Neither is there any evidence to show that treatment of normal antitoxic globulins with pepsin accords new specificity to these serum components. The new antigenicity which these investigators have observed does not, therefore, appear to be due to peptic digestion, nor to the process of purification, but rather to the nature of the original antitoxin molecule.

**h. Crystalline Diphtheria Antitoxin Antigenically Distinct from Normal Serum Components.** The inability of the previous investigators to differentiate the antigenic specificity of the antibody from that of normal globulin may perhaps be attributed to the fact that even the antibodies precipitated with their homologous antigens contain as integral portions of their molecules components indistinguishable from normal serum globulins. It has been shown by Parfentjev (1936, 1938), Pope (1938, 1939), and others that treatment of diphtheria antitoxin with pepsin under certain conditions splits the antitoxin into an active and an inactive portion. This observation has been

substantiated by Pope and Healey (1938, 1939) with ultracentrifugal experiments. Pope further showed that after treatment of antitoxic pseudoglobulin with pepsin at pH 4.2, the antitoxic product is no longer coagulable at 58°C., whereas the inactive split component is completely coagulable at this temperature. Peterman and Pappenheimer (1941) studied the physico-chemical properties of an antitoxic pseudoglobulin preparation and found it to be homogeneous by sedimentation, electrophoresis and diffusion criteria. Its molecular weight (184,000) was nearly the same as that of normal horse pseudoglobulin, but its mobility was different from those of any of the protein components of normal serum. This antitoxic pseudoglobulin contained 86,000 antitoxic units per gram, and was only 43.5 per cent specifically precipitable by toxin. After digestion with papain at pH 4.2 and the coagulation of the split product at 58°C. the antitoxic material remaining in the supernatant was almost homogeneous. Its molecular weight now was 113,000 and it contained 135,000 antitoxic units per gram.

In a comprehensive study Northrop (1942) reported the isolation of crystalline diphtheria antitoxin from trypsin digested antitoxic material. The crystalline antitoxin has a molecular weight of 90,000, which is very nearly one-half of the molecular weight of the original antitoxic horse serum pseudoglobulin, and smaller than the antitoxin obtained by Peterman and Pappenheimer. The antitoxin of Northrop was strictly homogeneous in the ultracentrifuge with a sedimentation constant of  $5.7 \times 10^{-13}$  (Rothen, 1942). The material showed only one boundary in the electrophoresis cell at pH 7.3 or 3.0. It was 90 per cent or more precipitable by diphtheria toxin and had about 700–900 antitoxic units per milligram protein nitrogen by the flocculation test and about 700 units per milligram by the animal test.

Immunologically the crystalline antitoxin was found to behave as an antigenic entity distinct from the normal horse serum proteins (Ten Broeck). The serum of a rabbit immunized against normal horse serum gave a precipitate with 1/4000 ml. normal horse serum (containing about 0.002 mg. of protein nitrogen) but gave no precipitate with 1 ml. of a solution of purified antibody containing 1/10 mg. of protein nitrogen.

Guinea pigs sensitized by the subcutaneous injection of purified antibody containing 0.003 mg. N gave a typical anaphylactic reaction

three weeks later when antibody protein containing 0.05 mg. N was injected intravenously. Similarly sensitized guinea pigs failed to react to normal horse plasma diluted to 1:10, but four out of six reacted to normal undiluted plasma containing 0.5 mg. of protein nitrogen, which probably, as stated by Northrop, indicates the presence of minute amounts of normal protein in the purified antibody preparation.





### Part III

## Antibody as a Specific Enzyme Inhibitor

THE THEORY formulated in this treatise considers humoral immunity as resulting from a biocatalytic process. This theory links the antigens with enzymes. As discussed in Part I, the antigenicity of all the simple and conjugated proteins is due to the catalytic activity of the protein molecule proper.\* Similarly the catalytic activity of simple protein enzymes such as pepsin, trypsin, urease, d-ribonuclease, etc., and that of conjugated protein enzymes such as heme-, copper-, alloxazine-, thiamine pyrophosphate- and pyridine-proteins is dependent on the protein molecule proper. The prosthetic groups *per se* (haptens of conjugated antigens, and coenzyme groups of conjugated protein enzymes) are incapable of catalytic activity either as antigens or as enzymes. In both instances the protein molecule, as part of the antigen or the enzyme must therefore be looked upon as the basic biocatalytic unit. *Since practically all proteins are antigenic the conclusion appears to be inescapable that all proteins are endowed with catalytic activity of the particular kind under discussion.†*

There is no doubt that there will be arguments raised against the inclusion of antigens among the enzymes. Such protests, however, must

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\*By definition an antigen must exhibit two properties: (1) the power of stimulating the production of antibody *in vivo*; (2) the power of combining specifically with this homologous antibody. Antigenicity has been ascribed to materials which give *in vitro* precipitation of complement fixation (Wassermann "antigen") but do not produce *in vivo* measurable antibodies. This loose usage of the term "antigen" may cause some confusion. In this treatise the term "antigen" implies the original definition as given above.

There may be non-protein substances which may prove to be truly antigenic, e.g. acetylated polysaccharide. If this does occur it is difficult to know whether the antigenicity is due to this polysaccharide alone or due to the combination of this polysaccharide and an *in vivo* protein to form a complete antigen.

†Bergmann (1938) stated that "The essential substances transmitted from one generation of cells to the next must be enzymes, and that they have to be enzymes gifted with the capability of synthesizing individual proteins by predetermined sequence of specificity reactions. . . . Therefore the proteinases owe their existence

find their answer in the concept as to what constitutes an enzyme. If we confine the term enzyme to certain familiar substances of biological origin capable of catalytically breaking down *in vitro* or *in vivo* certain selected substances, or capable of synthesizing higher complexes out of simple substrates, we will be setting artificial barriers or placing the known enzymes as an exclusive class behind closed bars. If on the other hand, we define an enzyme (or an antigen) as any protein capable of performing a specialized physiological function in accordance with the well known criteria of ideal catalysis, a comprehensive theory of biocatalysis is provided which links antigens, enzymes, vitamins and hormones\* and possibly other still unknown substances of similar rôle. The knowledge gained from the study of one family of biocatalysts will be useful in elucidating the mechanism of certain unexplained processes associated with other biocatalysts.

#### A. NATURE OF THE ANALOGY BETWEEN IMMUNE AND ENZYME REACTIONS

For over half a century immunologists have observed an analogy between immune and enzyme reactions. This analogy has been based on the fact that immune bodies react with antigens or their parts (haptens) with a high degree of specificity which favorably compares with the specificity exhibited between enzymes and their substrates.

The origin of the above cited analogy between immune and enzyme reactions goes back to as early as 1890. During this period notable advances were made in the study of the specificity of immune reactions, chemistry of proteins, carbohydrates, and the enzyme reactions by Emil Fischer, Behring, Ehrlich, Bordet, Calmette, Kitasato and many other investigators. In 1890 Behring discovered that the serum of an animal immunized against diphtheria was capable, when injected into a fresh animal, of conferring immunity upon the latter, which, failing the use

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to the preëxistence of other proteinases. There is, in life, a practically endless sequence of sequence reactions, in which one proteinase synthesizes the next by a predetermined reaction, and so forth. The sequence breaks off whenever a proteinase has synthesized a protein that does not possess enzymatic properties."

The definition, as suggested by us, embracing the versatile properties of the protein molecule is not in strict agreement with the implications suggested by the last sentence of the above quotation from Bergmann.

\*The reader is referred to an excellent review on the studies of the antigenic properties of hormones by Thompson (1941).

of immune serum, died from the effects of diphtheria toxin it received. Similar results were obtained with tetanus by Behring and Kitasato.

In 1891 Ehrlich treated animals with increasing doses of ricin and abrin and found that the toxin was neutralized *in vitro* when added to the treated animal's serum, proof of neutralization being offered by the fact that when certain proportions of toxin and immune serum were mixed *in vitro*, these mixtures were innocuous when injected into animals. He proved that the neutralizing action of immune serum upon each of these toxins was specific, that is, the antiserum for abrin did not neutralize ricin, and vice versa. To Ehrlich was also reserved the elucidation of the nature of the acquired immunity to toxins of snakes through the formation of antitoxins in the bodies of the toxin-treated animals, though the immunization against the toxins of snake venom had already been practiced by Sewall (1887), Calmette (1894), and by Fraser (1895).

These observations led Ehrlich to the conclusion that "the power of toxin to combine with antibodies must depend upon a specific atom-group in the toxin-complex possessing a maximal specific relation to definite atom-groups of the antitoxin complex, so that it rapidly unites therewith, like a lock and key," a figure borrowed from Emil Fischer in describing the action of specific ferments.\*

The observed striking analogy between the specificities of immune and enzyme reactions must have made a deep impression on the minds

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\*Pasteur found that *Penicillium glaucum* decomposed d-tartaric acid and left the l-tartaric acid intact. E. Fischer (1894, 1898) observed that yeast fermented d-hexoses, i.e., glucose, mannose, galactose and fructose, but not the l-hexoses;  $\alpha$ - and  $\beta$ -methyl-d-glucosides were hydrolyzed by enzymes; in contrast  $\alpha$ - and  $\beta$ -methyl-l-glucosides were not attacked. Whilst  $\alpha$ -methyl-d-glucoside was hydrolyzed by  $\alpha$ -glucosidase (maltase) only,  $\beta$ -methyl-d-glucoside was attacked by emulsin.  $\beta$ -d-Glucoside was hydrolyzed by  $\beta$ -d-glucosidase, and  $\beta$ -d-galactoside by  $\beta$ -d-galactosidase. This was explained by the fact that glucose and galactose differed stereochemically from each other at the 4-carbon atom. These facts led E. Fischer to believe that there existed a specific enzyme for each substrate. Maltose was believed to be hydrolyzed only by maltase, and in fact, glucoside, disaccharide, trisaccharide and polysaccharide were each believed to be attacked by a specific enzyme. The specific behavior of an enzyme towards the optical antipodes he believed to be conditioned by the optical configuration of the enzymes, which was compared with the lock-and-key system; that is, it can function only when it fits the lock.

Recent observations, however, have shown that  $\alpha$ -glucosidase of bottom beer yeast is capable of hydrolyzing  $\alpha$ -methylglucoside and maltose ( $\alpha$ -glucosido < 4-glucose) (Willstätter, Kuhn and Sobotka, 1924). Likewise saccharase, hydrolyzing saccharose, is capable of hydrolyzing the fructose linkage of raffinose ( $\beta$ -d-fructose < >  $\alpha$ -glucose >  $\alpha$ -galactose) (Kuhn, 1923). Emulsin from sweet almond hydrolyzes  $\beta$ -d-glucoside and its 6-brom-hydrin derivative,  $\beta$ -d-isorhamnoside,  $\beta$ -d-xyloside,  $\beta$ -d-galactoside

of the investigators of that early period and must have stimulated research along these lines. The early literature would seem to reveal the fact that these investigators experimented on the theory that the antitoxins played the rôle of specific enzyme inhibitors. For during this period, immunologists and enzyme chemists discovered numerous "normal" antitoxins, hemagglutinins and hemolysins, as well as naturally occurring enzyme inhibitors. Along with these findings, numerous investigators concerned themselves with the production of antibodies against known enzymes, using enzymically active preparations as antigens. They are: *anti-emulsin* (Hildebrandt, 1893), antiserum inhibiting the liquefaction of gelatin by staphylococcus and *B. pyocyaneus* (Von Dungern, 1899), *anti-rennin* (Morgenroth, 1899; Korschun, 1902), *anti-cyanurase* (Morgenroth, 1900), *anti-trypsin* (Achalme, 1901), *anti-coagulin* (Wendelstadt, 1901; Bordet and Gengou, 1901), *anti-inulase* (Saiki, 1907), antiserum inhibiting the production of pigment by *pyocyaneus* (Gheorghievsky, 1899), *anti-blastic immunity* against anthrax bacillus (Ascoli, 1908), anti-serum inhibiting the pneumococcal enzymes (Dochez and Avery, 1916), and antiserum inhibiting the formation of methemoglobin by pneumococcus (Cole, cited by Dochez and Avery, 1916).

The above citations show that during the period of 1890-1907, along with the pioneering observations on the toxin-antitoxin reactions, there was considerable activity in the study of anti-enzyme immunity. Unfortunately researches in this direction slowed down to such a negligible rate that until a decade ago investigations reminiscent of the activity of the earlier workers are rarely encountered. There has lately come about a renewed interest in anti-enzyme immunity due, probably, to the isolation of known enzymes in crystalline form. The recent findings with crystalline enzymes have established, beyond doubt, the antigenicity of enzymes. In fact the antibody against urease has been shown

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and  $\alpha$ -l-arabinoside. There is no evidence as to the presence of individual enzymes for each of the above substrates. A  $\beta$ -d-glucosidase is believed to be responsible for the hydrolysis of all of them (R. Weidenhagen, 1932; Helferich, 1933).

According to Weidenhagen (1940) there are only glucosidases, the principal specificity of which is confined to the constitution and configuration of the glycosidic sugars. The nature of the glucosidic pairs, whether sugar or aglycon, for the hydrolytic process as such are unessential, and exercise only a relative degree of specificity, particularly on the rate of hydrolysis. For instance, the ratio of speeds of hydrolysis between phenyl- $\beta$ -d-glucoside and phenyl- $\beta$ -d-xyloside is 150 to 1; that of saligenin- $\beta$ -d-galactoside to methyl- $\beta$ -d-galactoside is 60 to 1. It is stated by Weidenhagen that "there is no special key for each lock but a master key for a group of locks."

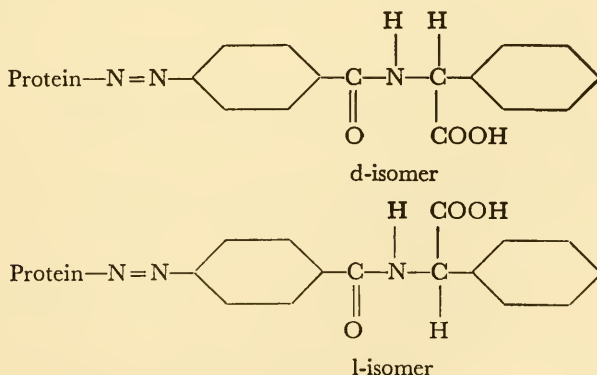


by Sumner and his associates to affect urease in a manner comparable in every respect to those exercised by antitoxins on toxins of various origin.

## 1. Serological Specificity of Stereoisomeric Conjugated-Protein Antigens

Since the statement made by Ehrlich that the specificity of toxin-antitoxin reactions is analogous to the specificity of fermentation reactions there has not been any systematic study to define clearly what this analogy consists of. During the last two decades the investigations with artificial conjugated antigens have yielded valuable data which have defined satisfactorily the rôle of determinant (prosthetic) groups on the specificity of the reactions with these antigens. Pioneering work in this field has been carried out by Landsteiner and his associates, and Avery, Goebel, and associates. Landsteiner (1936) formulated his findings as follows: 'There are striking differences among aromatic compounds, isomeric with respect to the position of substituents in the benzene ring, and the gradation in their cross reactions is to be taken as a definite indication that spatial structure, as well as chemical constitution in the ordinary sense of the word, plays an important role in serum reactions, "as had been shown for enzymatic processes by E. Fischer, and frequently confirmed. Hypothetically, Fischer's conception had been applied by Ehrlich to the investigation of serum reactions."

On immunizing animals with azo-proteins of d- and l-phenyl-(para-aminobenzoylamino)-acetic acids:

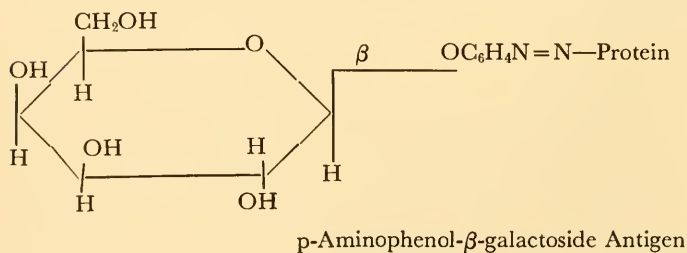
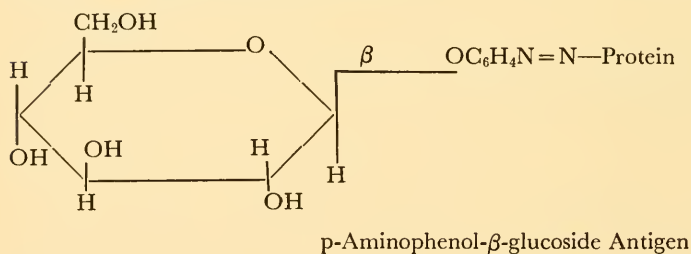




Landsteiner and van der Scheer (1928) obtained two different anti-sera which distinguished between the two isomeric antigens, demonstrating that a change in the spatial arrangement of atoms or radicals linked to one asymmetric carbon atom had resulted in the alteration of serological specificity of antigens. They also demonstrated that d- and l-para-aminotartranilic acids coupled with proteins stimulated the formation of antibodies which reacted distinctively with stereoisomeric antigens. There was practically no cross-reaction. The l- and d-immune sera gave rather weak group reactions with the meso-antigen. The m-immune serum gave practically no group reactions against l- and d-antigens.

Tartaric acid anti-sera also reacted with d- and l-maleic acid antigens, the d-serum chiefly with d-, the l-serum with l-compound, in agreement with the configurational correspondence demonstrated in the optical activity (rotation of polarized light in the same direction). On the basis of these and numerous other similar findings Landsteiner frequently referred to the analogy, as stated above, between immune and enzyme reactions.

Avery, Goebel, and associates published a series of papers dealing with the influence of spatial configuration of synthetic sugar-protein antigens on the specificity of serological reactions. Avery and Goebel (1929) prepared the azoprotein of p-aminophenol- $\beta$ -glucoside and p-aminophenol- $\beta$ -galactoside:



These hexosides were diazotized and coupled, respectively, with horse serum globulin and crystalline egg albumin.

Glucoglobulin antiserum prepared by the immunization of rabbits reacted not only with glucoglobulin but also with glucalbumin. Anti-serum reactive with glucalbumin showed no reaction with galactalbumin. The serum of rabbits immunized with galactoglobulin reacted with the solutions of galactoglobulin and galactalbumin in approximately equal titre. These antibodies, however, failed to precipitate glucalbumin. These data provided proof that the configuration of the sugar radical, regardless of the nature of the protein to which it was attached, was a determining factor in the serological specificity of these conjugated antigens.

The antisera prepared with sugar proteins also contained anti-protein antibodies which exhibited the protein specificity of the species from which they were derived. They could be removed from the immune sera by specific absorption without loss in the titre of the coexisting anti-carbohydrate antibodies. Conversely, the anti-carbohydrate antibodies could be specifically inhibited from reacting by the addition of the homologous glucoside without diminishing the activity of the anti-protein precipitins present in the same serum.

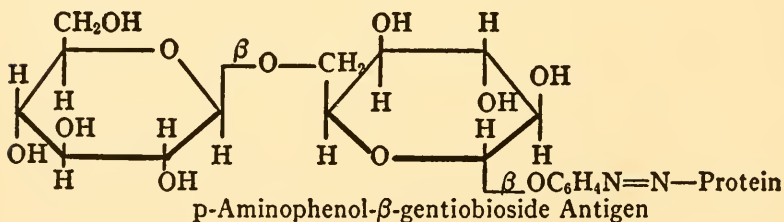
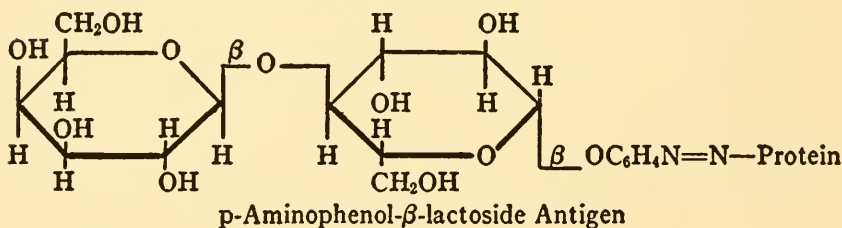
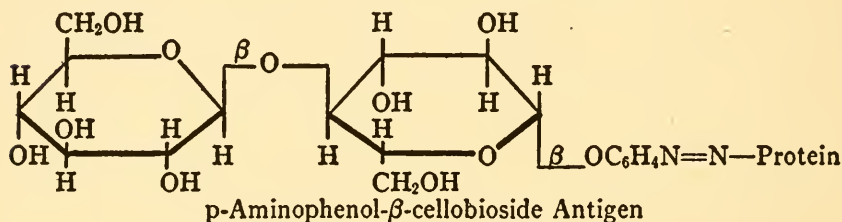
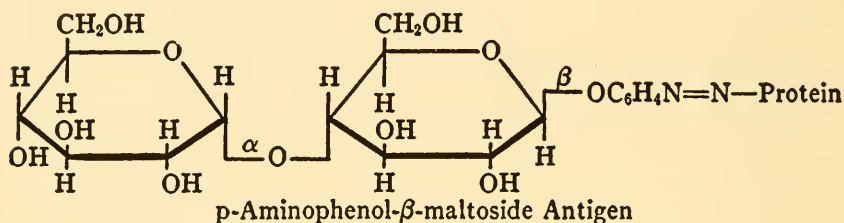
Avery, Goebel and Babers (1932) also showed that, though the antisera against azo-proteins of *p*-aminophenol- $\alpha$ -glucoside cross-reacted with  $\beta$ -antigen, a differentiation in the specificity of  $\alpha$ - and  $\beta$ -antigens could be affected by cross-inhibiting tests. The reaction of an immune serum with its homologous antigen was specifically inhibited only by the homologous glucoside, while the cross-reaction between this serum and the heterologous antigen was completely inhibited by either glucoside. This lack of reciprocal inhibition of the precipitins in  $\alpha$ - and  $\beta$ -antisera was interpreted, by the authors, as further evidence of the lack of the immunological identity of the two isomeric glucosides.

In studies with azo-proteins of  $\alpha$ -maltoside,  $\beta$ -cellobioside,  $\beta$ -lactoside and  $\beta$ -gentiobioside, Goebel, Avery and Babers (1934) demonstrated that the specificity of serological cross-reactions of disaccharide antigens is determined by the stereochemical configuration of the terminal hexose molecule; that only the test antigen containing the maltoside (terminal hexose,  $\alpha$ -glucose\*) reacted only in  $\alpha$ -antiserum.

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\*Strictly speaking the terminal hexoses in the above disaccharides are no longer free molecules and therefore should be named hexosides.

The two test antigens containing the cellobioside and gentiobioside (terminal hexose,  $\beta$ -glucose) reacted only in  $\beta$ -antiserum, and finally the lactoside test antigen (terminal hexose,  $\beta$ -galactose) reacted only in  $\beta$ -galactoside antiserum.



## 2. Specificity of Enzymes Which Catalyze Stereoisomeric Substrates

The above cited facts may be considered some of the most striking examples as characteristic of the rôle of determinant groups in general, and for the highly specific influence of the spatial configuration of glucosides in particular, on the specificity of immune reactions. These findings have shown beyond doubt the sensitivity of the response of the cytoplasm-antigen which is involved in the synthesis of the immune globulins. These facts have no doubt been instrumental in the emphasis of the oft referred analogy between immune and enzyme reactions. This analogy is clearly shown in the animal system which demonstrates enzyme specificity in the synthesis of a particular optical isomer as well as in the production of specific antibodies against optical antipodes. The analogy between these two types of biocatalytic reactions offers indeed a very attractive prospect of comparing directly the rôle of the participants in immune reactions with those of enzyme reactions.

In comparison with the rôle of the stereochemical and optical configuration of the carbohydrate groups of the synthetic gluco-protein antigens on the specificity of antibodies, we will discuss below the specificity of carbohydrases. According to the specificity theory of Weidenhagen (1932, 1940) the specificity of carbohydrases is characterized by their ability to hydrolyze or catalyze carbohydrates of three structural types. They are:

a. **Sugar Isomerism**, that is, the sugars differing at C-atoms. For example, the difference between glucose and galactose at the fourth carbon-atom;

b. **Constitutional Isomerism**, that is, the isomerism between aldoses and ketoses. For example, the difference between glucose and fructose;

c. **Ring Isomerism**, or  $\alpha$ - and  $\beta$ -isomerism.

For discussion of the possible analogy between the specificity of the glycoside—azoprotein antigens and the specificity of the carbohydrases which hydrolyze di- and tri-saccharides, the following table is constructed.

Table IV shows that a disaccharide such as saccharose containing  $\alpha$ - and  $\beta$ -hexosides are hydrolyzed by both the  $\alpha$ - and  $\beta$ -glucosidases. Each glucosidase can attack, however, only one of the linkages and

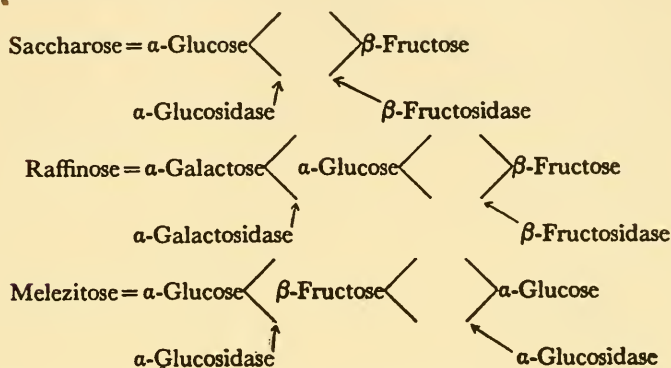
TABLE IV  
 *$\alpha$ - and  $\beta$ -Specificity of Glucosidases*

Glycosides	Hydrolyzed by:
<i><math>\alpha</math>-Glycosides</i>	
$\alpha$ -Heteroglucoside (hetero=aglucon, a non-sugar group)	$\alpha$ -glucosidase
Maltose= $\alpha$ -glucosido-4-glucose	$\alpha$ -glucosidase
Saccharose= $\alpha$ -glucoside- $\beta$ -fructoside	$\alpha$ -glucosidase and $\beta$ -fructosidase
Turanose= $\alpha$ -glucosido-fructoside	$\alpha$ -glucosidase
Melezitose= $\alpha$ -glucosido- $\beta$ -fructosido- $\alpha$ -glucoside	$\alpha$ -glucosidase
<i><math>\beta</math>-Glycosides</i>	
$\beta$ -Heteroglucoside	$\beta$ -glucosidase
Cellobiose= $\beta$ -glucosido-4-glucose	$\beta$ -glucosidase
Gentiobiose= $\beta$ -glucosido-6-glucose	$\beta$ -glucosidase
Salicin= $\alpha$ $\beta$ -glucoside (glucose+saligenin)	$\beta$ -glucosidase
<i><math>\alpha</math>-Galactosides</i>	
$\alpha$ -Heterogalactoside	$\alpha$ -galactosidase
Melibiose= $\alpha$ -galactosido-6-glucose	$\alpha$ -galactosidase
Raffinose= $\beta$ -fructosido- $\alpha$ -glucosido-6- $\alpha$ -galactoside	$\alpha$ -galactosidase and $\beta$ -fructosidase
<i><math>\beta</math>-Galactosides</i>	
$\beta$ -Heterogalactoside	$\beta$ -galactosidase
Lactose= $\beta$ -galactoside-4-glucose	$\beta$ -galactosidase
<i><math>\beta</math>-Fructofuranosides</i>	
Saccharose	$\beta$ -fructosidase (invertase)
Raffinose	$\beta$ -fructosidase
Gentianose= $\beta$ -fructosido- $\alpha$ -glucosido-6- $\beta$ -glucoside	$\beta$ -fructosidase
Inulin= $\Delta$ polymer of $\beta$ -fructose	$\beta$ -fructosidase

Glucose and galactose in the above combinations possess pyranose structure and fructose furanose structure, and all have d-configuration.



not the other.  $\alpha$ -Glucosidase will attack the  $\alpha$ - and not the  $\beta$ -linkage and  $\beta$ -fructosidase will attack the  $\beta$ - and not the  $\alpha$ -linkage in saccharose. This is illustrated in the following manner:



In this connection it may be mentioned that an enzyme from sweet almond capable of hydrolyzing  $\beta$ -d-glucuronide will not hydrolyze  $\beta$ -d-glucoside. It is therefore clear that in this case  $\beta$ -configuration is not the only essential condition for the specific action of an enzyme. Similarly,  $\beta$ -glucosidase will hydrolyze  $\beta$ -glucoside and not  $\beta$ -galactoside. Hypothetically it is assumed that  $\beta$ -glucosidase might hydrolyze at different rates both  $\beta$ -glucoside and  $\beta$ -galactoside, which might be interpreted to indicate a relative specificity of  $\beta$ -glucosidase towards  $\beta$ -galactoside. The evidence for this assumption, however, is still very weak (Helferich, 1933, 1938).

### 3. Comparison of the Stereoisomeric Specificities of Immune and Enzyme Reactions

Comparing the specificity of the reaction between an antibody and the homologous antigen containing a glucoside with the specific action of a glucosidase on a glucoside substrate, the following facts are evident. As previously suggested by others (Marrack, 1938) we will arbitrarily compare the antibody with the enzyme, and the antigen with an enzyme substrate. Avery and Goebel (1929), and Goebel, Avery and Babers (1934) showed that the antiserum against  $\beta$ -glucoside did not react with the antigen containing  $\beta$ -galactoside; and the antiserum against the  $\beta$ -galactoside did not react with the test antigen containing  $\beta$ -glucoside. Table IV likewise shows that the enzyme  $\beta$ -

glucosidase will not hydrolyze  $\beta$ -galactoside; conversely  $\beta$ -galactosidase will not hydrolyze  $\beta$ -glucoside.

These facts are compared below in a tabulated form:

TABLE V

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Maltoside:

- (a) As test antigen (with  $\alpha$ -glucose as terminal hexose\*) reacted only in  $\alpha$ -antiserum
- (b) As enzyme substrate (with  $\beta$ -glucose as terminal hexose) is hydrolyzed only by  $\alpha$ -glucosidase

Cellobioside:

- (a) As test antigen (with  $\beta$ -glucose as terminal hexose) reacted only in  $\beta$ -antiserum
- (b) As enzyme substrate (with  $\beta$ -glucose as terminal hexose) is hydrolyzed only by  $\beta$ -glucosidase

Gentiobioside:

- (a) As test antigen (with  $\beta$ -glucose as terminal hexose) reacted only in  $\beta$ -antiserum
- (b) As enzyme substrate (with  $\beta$ -glucose as terminal hexose) is hydrolyzed only by  $\beta$ -glucosidase

Lactoside:

- (a) As test antigen (with  $\beta$ -galactose as terminal hexose) reacted only in  $\beta$ -galactoside antiserum
  - (b) As enzyme substrate (with  $\beta$ -galactose as terminal hexose) is hydrolyzed only by  $\beta$ -galactosidase
- 

\*Strictly speaking the terminal hexoses in the above disaccharides are no longer free molecules and therefore should be named hexosides.

The nature of the factors governing the specificities of the above cited artificial gluco-protein antigens can be summarized as follows: The antigenic specificities are confined to: (a) the structural configurations of hexose molecules, such as glucose and galactose, (b) the  $\alpha$ - and  $\beta$ -configurations of the glycosides, such as  $\alpha$ - and  $\beta$ -azophenol glycosides, and (c) the configurations of the terminal hexosides such as  $\alpha$ -glucoside in  $\beta$ -maltoside,  $\beta$ -glucoside in  $\beta$ -cellobioside, and  $\beta$ -gentiobioside, and  $\beta$ -galactoside in  $\beta$ -lactoside. In this connection it is also to be noted that the specificities of the action of enzymes on particular substrates (compared with the above carbohydrates) is governed by the above configurational differences.

The above comparison shows that there is apparently a perfect

similarity between the specificity of these antigen-antibody reactions and that of the action of these enzymes on their specific substrates. This excellent analogy would have served as a basis for a clearer understanding of the immune reactions if it were at all possible to compare antibodies to enzymes and antigens to substrates. As will be discussed below this apparent similarity, or the analogy recognized long ago, would appear to have been based on a faulty interpretation of facts. At this point it suffices to state that the active groups in the above antigens can function, either as a directive force in catalyzing the synthesis of the specific antibodies, or as a specific point of attack by enzymes, i.e., hydrolysis of  $\alpha$ -glucoside by  $\alpha$ -glucosidase, etc. From the standpoint of antigenic specificity (and of enzyme action) of these groups it is the first function, and not the second, which is of critical importance.

In a recent article Marrack (1938a) discussed the subject of "Immunochemistry and Its Relation to Enzymes." Another paper dealing with this subject in a less specific manner was written by Westphal (1940). From the standpoint of subject matter the paper by Marrack will be considered here. Since the point of view with which Marrack discusses this subject has a direct bearing on the validity of his conclusions, we feel justified in presenting his views in some length by introducing the following quotations from his article:

"It would be better if it were possible to study the effects of variations of the chemical structure of both partners in enzyme reactions and immunity reactions on the affinity between them. However, in each case only one partner—the substrate in an enzyme reaction and antigen in an immunity reaction—can be varied as we wish; in general we have to take enzymes as they occur naturally, and antibodies as they are formed in response to our injections. *We are left to infer that the specificity of antibody and enzyme is determined by details of chemical structure to some extent comparable to those that determine the specificity of antigen and substrate.\** When we compare antigen and substrate in respect of the relation of their specificity to their chemical structure it does not mean that antigen and substrate are analogous partners in the two paired reactions; probably antigen would be better compared with enzyme. It is merely that in these two partners it is possible to vary the chemical composition," and further: "Hitherto we

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\*"Italics" by M. G. Sevag.

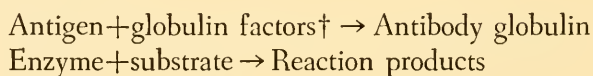
have considered antigens as if they were analogous to the substrates in enzyme reactions. This is to some extent justifiable as relatively small molecules can act as substrates and combine with antibodies; whereas antibodies and apparently all enzymes consist of large molecules. It is also convenient since the effect on specificity of chemical changes in antigen can be compared with the effect of similar changes in substrates. But it may be more correct to regard antigens, whose action is to produce some change in antibodies that results in insolubility, as analogous to enzymes."

For the sake of historical interest, the concept presented by Wells (1929) in his book on the *Chemical Aspects of Immunity* may be briefly mentioned at this point. After summarizing the known facts regarding the resemblances of the physico-chemical properties of toxins and enzymes, Wells writes as follows:

"Enzymes and toxins seem to produce their effects according to different laws:—A small amount of enzyme can in course of time produce an almost indefinite amount of effect, whereas toxins act more nearly quantitatively.\* It seems as if the enzyme were bound to the body upon which it acts, as is the toxin, but that after it has destroyed this body it is set free in a still active form ready to accomplish further work; whereas the toxin is either not set free, or it becomes inactive after it has once been combined."

#### 4. Immune and Enzyme Reactions—A Comparison of Reaction Mechanisms

The comparison of the two systems can be schematically diagrammed as follows:



It is apparent from the above schema that in the *in vivo* mechanism the following pairs have the same rôles:

- (a) Antigen and Enzyme
- (b) Globulin Factors<sup>†</sup> and Substrate
- (c) Antibodies and Reaction Products

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\*Combination between toxin and antitoxin.

<sup>†</sup>The term Globulin Factors does not stand for normal globulins. It stands for peptides or amino acids which are utilized for the synthesis of antibody globulins.

The specific catalytic action of antigens on globulin factors or a certain group of amino acids, or polypeptides, etc., produces the antibody globulin molecule. Antibody is therefore a final reaction product with specific affinity for the antigen. Similarly the specific catalytic action of an enzyme on its substrate produces final reaction products with specific inhibitory affinity for the enzyme.

Antibodies being final reaction products of a chain of catalytic reactions—associated with the synthesis of globulin—should be looked upon as specific inhibitors of antigens. As inhibitors of antigens, or as inhibitors of the biological activity (toxicity, etc.) of antigens they are comparable to the specific enzyme inhibitors which result from the action of enzymes on substrates. This is particularly true when the enzyme reactions take place in a complex protein environment as shown by Northrop (1922) and others.

The combination between an antigen and its homologous antibody is a stable one and usually results, under optimal conditions, in a precipitation or agglutination reaction. This reaction is reversible under certain not too mild conditions, such as heating at 50°–60°C., dissociation by 10 per cent sodium chloride or the separation of the antibody from its combination with antigen by dilute acids. The action of antibody on antigen does not produce a permanent chemical change in the latter. The neutralization of the toxic properties of toxins and venoms is not one of destruction but is a process of blocking of active groups in the same way that an inhibitor blocks enzymically active groups. Marrack's contention that the action of antigens on antibodies is to produce some change in antibodies resulting in insolubility concerns a secondary and physico-chemical property of the antigen-antibody complex rather than a change produced in the antibody molecule. For antibodies freed from their combination with antigens have been shown to exercise a neutralizing property identical in every respect with that exercised previous to their combination with antigen. The stable union between an antibody and antigen is therefore a stoichiometrical reaction representing a simple combination between multivalent radicals, and it obeys the mass action law, as often emphasized by Heidelberger and Kendall (1935; Heidelberger, 1939; Kendall, 1942).

In contrast, an enzyme molecule does not form a *stable* combination with its substrate. The life of the combination between an enzyme



substrate is often less than one millionth of a second. That is, in one second more than one million molecules come into contact with a single enzyme molecule during which time they are catalyzed and metabolized. Such a continuous combination, dissociation and destruction of the substrate at tremendous speed by its specific enzyme is in no way comparable to the antigen and antibody reaction. It can be seen readily that the action of an enzyme on its substrate is dynamic and a catalytic reaction, and that of antigen with antibody is non-catalytic and results in a static state. This process has a true counterpart in the enzyme reactions.\*

In practically all enzyme reactions whether the substrates are anabolized or catabolized by the action of enzymes, certain inhibitory

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\*In *in vitro* experiments the combination between an antigen and antibody may or may not result in precipitation. If the number of antigen molecules is in large excess a precipitation may fail to occur (certain bacterial carbohydrates or small molecular weight proteins reacting with their respective antibodies). However, there are antigen-antibody reactions in which precipitation occurs even in the region of excess antigen (a large molecular weight protein antigen reacting with its specific antibody). In inhibition experiments, reactions which involve the participation of antibody and of small molecular weight haptens, a precipitation fails to occur, despite the complete neutralization of the combining groups of the two reactants. The phenomenon of precipitation does not therefore accompany all the phases of, or various types of, antigen-antibody reactions.

The counterparts of the various phases of or of various types of antigen-antibody reactions are encountered in reactions involving a combination between an enzyme and its reaction products. These inhibitors combine with and completely inhibit the activity of the enzymes. Such inhibitions (or combinations), however, may or may not result in the formation of an insoluble enzyme-inhibitor complex. As in antigen-antibody reactions the failure to form an insoluble complex appears to depend on the molecular size of the inhibitor as well as the ratio of the number of molecules of the two reactants. As discussed below the enzyme pepsin is inhibited by pepsin inhibitor; the degree of inhibition is dependent on the concentration of the inhibitor. When, however, one molecule of inhibitor combines with one molecule of pepsin the enzyme is completely inhibited as a consequence of the formation of a soluble inhibitor-pepsin complex. As in certain antigen-antibody reactions, the combination between the inhibitor and pepsin does not result in a precipitation. On the other hand a solution of trypsin inhibitor mixed with a solution of trypsin of equal molecular strength completely inactivates trypsin with the formation of a crystalline compound with trypsin.

Straus and Goldstein (1943) have reported a study on the zone behavior of enzymes. Departing from the classical treatment of the kinetics of enzyme reactions, they show that under a number of common conditions systems involving the participation of an enzyme, specific substrate and an inhibitor, the enzyme-substrate, enzyme-inhibitor systems behave in three distinct ways depending upon the concentrations of the reactants and the dissociation constants of the system. An important practical consequence of the theory of zone behavior concerns the effect of diluting a mixture of enzyme and inhibitor (or substrate). They show that dilution is a crucial operation which significantly affects the subsequent experimental observations. For a fuller understanding of this study the reader is referred to the original article.

reaction products are formed in due course. By virtue of the structural similarity to the substrates from which they are formed, these products exercise specific affinity for the enzymes in accordance with the mass action law, in the same manner as antigens combine with antibodies. The degree of inhibition is dependent on the degree of affinity for the enzyme and the amount of the inhibitor produced. The consequences of the reaction of the enzyme molecule with its reaction product as inhibitor, is comparable in every respect with the reaction of antigen with the antibody which has resulted from the action of antigen *in vivo*. The inhibitor produced by one enzyme will not inhibit another enzyme. It is therefore highly specific in the same way that an antibody will not combine with a heterologous antigen.

## 5. Zinsser's View on the Formation and the Role of Antitoxins

Northrop (1922) in his studies on the kinetics of the action of pepsin and trypsin on protein observed certain divergences which he explained by the production of hydrolytic products acting as inhibitors. These inhibitory products combining with the enzyme maintained an equilibrium which obeyed the ordinary mass action law. The digestion of protein by pepsin, combined with such substances as peptone (the word "peptone" used broadly as signifying substances in solution with which the pepsin combines without hydrolyzing them), was not proportional to the total concentration of the pepsin. He therefore, believed that as the pepsin digested the protein, peptone-like substances are formed with which pepsin combined and with which it then maintained an equilibrium following the mass action law. In adding increasing amounts of peptone to pepsin solution, as a result of this manner of combination, the first amounts added inactivated more pepsin than the latter additions, which Northrop pointed out is in very striking analogy to the manner in which antitoxin and toxin react.

In the case of trypsin as it acted on the undigested protein solution trypsin inhibiting substances were also formed. The activity curve showed that, at first, there was a very rapid drop of activity which gradually became slower.

Hans Zinsser (1923) in his discussion of toxin and antitoxin stated that a good deal of light may be hoped for in regard to the nature of

antigen-antibody unions from investigations upon the nature of enzyme reactions. Discussing the above cited results of Northrop with a view as to the relation of such findings to the toxin-antitoxin reaction he made the following statement: "These experiments of Northrop do not, of course, solve the question of antigen-antibody unions, but they do serve to bring the analogy of toxin-antitoxin relations much closer to laws governing the union of enzyme with substrate. Moreover, they show that enzyme is actually used up in its reactions, just as toxin is used up in its reactions with antitoxin, and that equilibrium following the mass action law may be a definite factor in the quantitative relations governing the reactions. It is not at all impossible that the general laws which govern reactions between trypsin and its inhibiting substances are similar to those which govern the toxin-antitoxin reaction. Moreover, while it is a dangerous subject upon which to theorize, it is yet not utterly impossible that the toxins are closely analogous to enzymes, and that they produce in their action upon cells products of injury which, passing into the circulation, become the specific inhibitors of the toxin or the antitoxin. We do not wish to dignify this with the label of a theory, but in subjects as vague as the origin and biological meaning of antitoxins, we must grasp at every straw that may suggest experimental paths for enlightenment."

Since Zinsser expressed the above view two decades ago our knowledge of the mechanism of enzyme and immune reactions has greatly advanced. Numerous enzymes have been isolated in crystalline and highly pure form, and their chemical nature has been clarified. With the same pace the chemical nature of whole antigens, haptens and antibodies has been quite extensively studied. The reactions of antigen and antibody, principally through the studies of Heidelberger and Kendall, have been quantitated and related to the mass action law. Reëvaluation and correlation of these findings appear to lend support to the view expressed by Zinsser and define the nature of the analogy between immune and enzyme reactions observed long ago by Ehrlich.

## B. THE FORMATION OF SPECIFIC INHIBITORS IN ENZYME REACTIONS

As stated above, by the action of an enzyme on a substrate specific inhibitors, structurally related to the substrate, are generally pro-

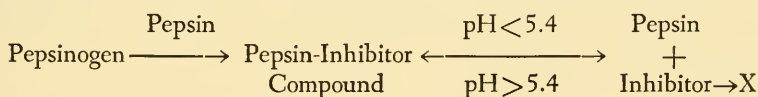
duced. This structural relationship between the substrate and the inhibitor is the reason for the affinity an inhibitor possesses for the enzyme, and thereby causes the inhibition of the enzyme. In the following pages the formation of several such specific enzyme inhibitors will be considered.

## 1. Pepsin Inhibitor

It has been shown by Herriott (1938, 1941) that pepsinogen autocatalytically is transformed into pepsin at pH 4.6; the pepsin formed catalyzes the reaction. During this transformation there are simultaneously produced certain polypeptides one of which has a powerful inhibiting action on pepsin at pH 5.0–6.0. The inhibitor has been isolated in the form of spheroids which change later on to rosettes of tiny needles. This inhibitor on combining with pepsin between pH 5.0 and 6.0 forms a dissociable *inhibitor-pepsin* complex. The reversible combination of pepsin with the inhibitor follows quantitatively the simple mass law equation.

The molecular weight of the inhibitor as determined by diffusion and combining equivalence with pepsin lies somewhere between 4000 and 10,000. Since 0.000,25 mg. of inhibitor nitrogen is approximately equivalent to 0.0012 mg. of pepsin nitrogen in the inhibitor-pepsin complex the ratio of mg. of inhibitor N to mg. of pepsin N is 1:4.8, or one molecule of pepsin reacts with one molecule of inhibitor.

This complex dissociates upon dilution and upon acidification (in a manner which appears to be comparable to the dissociation of antigen-antibody complex under the same conditions). It does not combine in acid solution. The entire reaction is presented by Herriott as follows:



The first reaction from pepsinogen to pepsin-inhibitor compound is catalyzed by pepsin, while the second reaction from the compound to free pepsin and the inhibitor is a reversible dissociation. The third reaction is the destruction of the inhibitor on long standing with pepsin between pH 2.0 and 5.0.

The pepsin inhibitor is a polypeptide which has basic groups which are exposed, since it is precipitated by many reagents used to precipi-



tate basic substances, namely, tungstic, phosphotungstic, flavianic, picric, and picrolonic acids. The inhibitor is not precipitable with trichloroacetic acid.

The inhibition of pepsin by this inhibitor is demonstrated by the rennet method (decrease in milk clotting activity of pepsin) which is carried out at pH 5.8.

The pepsin inhibitor has no demonstrable effect on the activity of crystalline trypsin, on the milk clotting activity of crystalline chymotrypsin or commercial rennet. Conversely, the crystalline trypsin inhibitor has no effect on the milk clotting action of pepsin. This indicates a high degree of specificity, that usually is associated with enzymes, also exists among some inhibitors of enzymes. Bovine pepsin was inhibited to the same degree as swine pepsin, but chicken pepsin was not inhibited at all. On the other hand, a crude inhibitor solution prepared from chicken pepsinogen inhibited both swine and bovine pepsin, but had no effect on the chicken pepsin.

According to Bourdillon (1945) the action of pepsin (and other proteolytic enzymes) on antitoxin (diphtheria) pseudoglobulin yields in addition to a heat labile and low molecular weight nitrogenous material, a new protein of reduced molecular weight (60 per cent of the native substance) which still has all the characters of a native substance. Split antitoxin is only slowly hydrolyzed by pepsin in moderately acid medium and is thus able to form reversible compounds of appreciable stability with pepsin. This complex contains from two to three molecules of pepsin to one of antitoxin. The split antitoxin-pepsin complex is markedly similar to the edestin-pepsin combination. In both cases, maximum precipitation occurs at about pH 4.0 and the two proteins combine with pepsin in approximately equal amounts. Both complexes are richer in pepsin when formed in the presence of excess pepsin.

## 2. Trypsin Inhibitor of Pancreatic Extracts

Kunitz and Northrop (1936) isolated the trypsin inhibitor from trypsinogen crystals. The inhibitor is believed to play a very important part in regulating the activation of trypsinogen, and in partly activated pancreatic extracts more or less active trypsin occurs in the form of an inactive compound with the inhibitor. Like pepsin inhibitor, the



trypsin inhibitor is a polypeptide with a molecular weight of 6000. It is likewise not precipitable with 2.5 per cent trichloroacetic acid, either hot or cold, nor by boiling in water. A solution of the inhibitor mixed with a solution of trypsin of equal molecular strength at pH 7.0 in 30 minutes at 6°C. completely inactivates trypsin. The inhibitor forms a compound with trypsin which is dissociable at pH 1.0. The inactivating effect of the inhibitor is demonstrated in experiments on the digestion of casein, the digestion of sturin, or the activation of chymotrypsinogen into chymotrypsin, or trypsinogen into trypsin in the presence of trypsin or salt, and the clotting of blood. The substance also inhibits chymotrypsin but to a less marked extent. One molecule of inhibitor combining with one molecule of trypsin results in the formation of inhibitor-trypsin complex in the form of hexagonal, many-faced crystals with a molecular weight of 40,000. This combination is split by trichloroacetic acid which precipitates trypsin and leaves the inhibitor in solution.

### 3. Trypsin Inhibitor of Blood Serum

It is known that normal serum also contains trypsin inhibitor which blocks the activity of trypsin. Schmitz (1938) isolated this inhibitor in various ways. On precipitating the serum proteins with trichloroacetic acid the inhibitor remained in the supernatant acid solution. It was separated by ultrafiltration from the proteins. After precipitation of serum proteins with acetone the inhibitor was found in the acetone solution. The behavior of this inhibitor against trypsin was analogous to the inhibitor isolated from pancreas by Kunitz and Northrop (1936).

### 4. Inhibition of Carbohydrases by the Reaction Products

A disaccharide or glucoside such as sucrose, with specific affinity for invertase, on hydrolysis yields reaction products which manifest inhibitory affinities for the enzyme. Thus fructose and glucose, the products of hydrolysis, inhibit invertase markedly (Henri, 1902; Michaelis and Menten, 1913).  $\alpha$ -Methylglucoside which has the same configuration as the  $\alpha$ -glucose is also found to inhibit invertase (Michaelis and Rona, 1914). On the other hand, those disaccharides and glucosides, such as maltose, lactose and  $\beta$ -methylglucoside, possess-

ing configuration different from sucrose, lack affinity for invertase and therefore are not hydrolyzed by invertase and do not inhibit the activity.

Kuhn (1925) and van Klinkenberg (1932) have further shown that the inhibition of an enzyme by its reaction product is highly specific, and that this specific inhibition is dependent on the configuration of the reaction product in the same manner that the serological specificity of the reactions between conjugated sugar-protein antigen and its homologous antibody is dependent on the configuration of the sugar molecule in the antigen.

Kuhn (1925) determined the inhibitory effect of stereoisomeric carbohydrates on the activity of various amylases. He found that  $\beta$ -amylase present in malt was inhibited most strongly by  $\beta$ -maltose. On the other hand  $\alpha$ -amylase present in takadiastase (also in pancreas) was inhibited by  $\alpha,\beta$ -maltose twice as strongly as by  $\beta$ -maltose. These findings are significant in view of the fact that  $\alpha$ -amylase hydrolyzing starch produces  $\alpha$ -maltose and  $\beta$ -amylase produces  $\beta$ -maltose as a reaction product (starch consists of 36 per cent  $\alpha$ -starch and 64 per cent  $\beta$ -starch). These data show that various amylases are most strongly inhibited by those maltoses which they themselves produce specifically.

The following table is constructed from the results obtained by Kuhn which show clearly that strong inhibition is caused by  $\beta$ -maltose and  $\beta$ -glucose in contrast to a negligible inhibitory effect exercised by their  $\alpha$ -isomers.\*

TABLE VI  
*Inhibition of Malt  $\beta$ -Amylase by Stereoisomeric Sugars*  
Per cent Inhibition by:

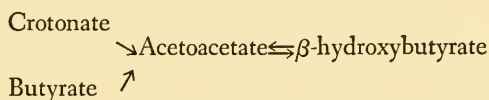
Substrate	$\alpha$ -Glucose	$\beta$ -Glucose	$\alpha$ -Maltose	$\beta$ -Maltose	$\alpha$ -, $\beta$ -Maltose
Soluble starch	5, 11*	37, 39	—	55, 50	31, 36
Amylose	4, 0.4	48, 44	2, 14	60, 49	40, 55

\*The 5 and 11, etc. pair of figures represent per cent inhibitions calculated from results obtained by Kuhn respectively after 12 and 25-minute reaction periods.

\*Hunter and Downs (1945) reported that the action of arginase upon arginine at pH 8.4 is inhibited by all  $\alpha$ -amino acids of the naturally occurring configuration, but not by  $d$ - $\alpha$ -amino acids, amino acids having the amino group in other than the  $\alpha$ -position, urea, or native proteins.

## 5. Inhibition of the Oxidation of $\beta$ -Hydroxybutyric Acid by Its Reaction Product Acetoacetic Acid and Related Acids

Jowett and Quastel (1935) found that fatty acids are oxidized at considerable rates by slices of liver *in vitro* and give as one of their oxidation products acetoacetic acid, as in the body. Jowett and Quastel (1935) studied the rate of acetoacetic acid production from butyric, crotonic and  $\beta$ -hydroxybutyric acid under various conditions. On the basis of the results obtained they proposed a mechanism of the oxidation of these acids to acetoacetic acid as follows:



They found that 0.001M benzoate, cinnamate and phenylpropionate strongly inhibit the oxidation of butyric and crotonic acids to acetoacetic acid. Cinnamate and propionate inhibit oxidation of  $\beta$ -hydroxybutyric acid to a much smaller extent than the oxidation of butyric and crotonic acids.

Green, Dewan and Leloir (1937) studied the nature of the enzyme system responsible for the oxidation of  $\beta$ -hydroxybutyric acid to acetoacetic acid. The dehydrogenase system was prepared from heart muscle, and coenzyme I was found to be an indispensable component of the system. This system specifically catalyzed the oxidation of l- $\beta$ -hydroxybutyrate to acetoacetate. They also showed that the change from  $\beta$ -hydroxybutyrate to acetoacetate is reversible. The highly specific nature of the dehydrogenase system was evidenced by the fact that it did not catalyze the oxidation of  $\beta$ -hydroxypropionic,  $\alpha$ -hydroxybutyric, crotonic,  $\gamma$ -hydroxybutyric, butyric and acetic acids.

The above specific dehydrogenase system was inhibited by 0.03 M iodoacetate, pyruvate and oxalacetate 56, 48 and 71 per cent, respectively. Under identical conditions the inhibition by 0.012 M of the reaction product, acetoacetic acid, was 55 per cent.

Green *et al.*, as cited above, appear to have shown that the tissue slices used by Quastel and his associates represent a mixture of en-

zyme systems capable of oxidizing several of the fatty acids mentioned above. In evaluating the inhibitions of the tissue enzyme systems it is clear that the inhibition of the oxidation of butyric and crotonic acids by benzoate, cinnamate and phenylpropionate, and relative absence of inhibition of the oxidation of  $\beta$ -hydroxybutyric acid by these inhibitors indicate their affinity for one enzyme system and not for the other. The inhibition of the specific oxidation of  $\beta$ -hydroxybutyric acid by the enzyme system used by Green, *et al.* by iodoacetate, pyruvate, oxalacetate, etc. as well as by the reaction product acetoacetic acid must be attributed to their common affinity for the same enzyme system. Such common affinities for an enzyme are exhibited by substances which are structurally similar to the specific substrates or their reaction products.

## 6. Inhibition of Succinoxidase by the Oxidation Product of Succinic Acid, and by Structurally Related Acids

That acids structurally related to a substrate and to the reaction products inhibit the particular enzyme system has been reported by other investigators. Weil-Malhebre (1937) reported that in the presence of an enzyme preparation from fresh ox heart succinic acid consumed (during a two-hour period) 311  $\mu$ l of  $O_2$ , 1- $\alpha$ -hydroxyglutaric acid, 10  $\mu$ l,  $\alpha$ -glycerophosphate, 29  $\mu$ l and d(-)glutamic acid 45  $\mu$ l; 1-(+) glutamic acid and  $\beta$ -glycerophosphate were unreactive. In agreement with the earlier observations of Quastel and Wooldridge (1928) he found that malonic acid\* inhibited the enzyme completely, whereas maleic acid did not inhibit at all.  $\alpha$ -Ketoglutaric acid (M/20) was found to inhibit specifically the succinic dehydrogenase 40–50 per cent. Other keto acids, such as pyruvic or 2-ketogluconic acids or other substances of related constitution, e.g., hydroxyglutaric or glutamic acids, exercised no inhibition.

Potter and Elvehjem (1937) studied the succinoxidase system of the brain, liver and kidney tissues of rats and chickens. The inhibitory

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\*That malonic acid is a biological metabolite has been demonstrated by Raistrick (1938). It is a component of, and is liberated by alkaline hydrolysis of a high molecular weight polysaccharide which is produced from glucose by a strain of *Penicillium luteum*. Vennesland and Evans (1944) reported that the oxidation by tissue of oxalacetic acid, a derivative of succinate, yielded malonate. Malonate, therefore, inhibits the very enzyme which produces it.

effect of 0.034 M oxalic, malonic, glutaric, adipic, l-aspartic, l-malic and fumaric acids on the oxidation of succinic acid was determined. Malonic and oxalic acids exercised most marked inhibition, while glutaric, adipic and aspartic acids inhibited about 25 per cent, and fumaric acid 24 per cent. The last is the first oxidation product of succinic acid.

In these cases also the inhibition of succinic acid oxidation by malonic acid would seem to be easily explainable on the basis of an affinity between succinic acid dehydrogenase and malonic acid, owing to the similarity of configuration. Since malonic acid cannot be dehydrogenated, unlike the activated succinic acid, it does not readily dissociate from the enzyme and consequently blocks the enzyme from reacting with succinic acid. The inhibition of succinoxidase by the other acids must likewise be attributed to its property of combining with compounds possessing affinities similar to succinic acid.

## 7. Inhibitory Effect of the Salts of Organic Acids on the Oxidation of Tyrosine by Tyrosinase

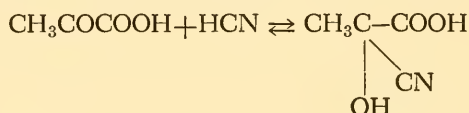
With the object of finding an analogy to the inhibition phenomenon observed in precipitin reactions with organic acids, isomeric with or structurally related to those coupled with proteins, Landsteiner and van der Scheer (1927) examined the effect of 41 sodium salts of organic acids on the action of the oxidase of potatoes and mushrooms on tyrosine (p-hydroxyphenyl- $\alpha$ -aminopropionic acid). In a general way they stated that carboxylic acids of benzene and other cyclic compounds acted more strongly than fatty acids, and that stronger inhibition was caused by the *meta* and *para* substituted acids than by the *ortho* substituted.

## 8. Inhibition of Lactic Acid Dehydrogenase by the Reaction Product Pyruvic Acid

Green and Brosteaux (1936) in their study of the mechanism of the dehydrogenation of lactic acid by enzyme solutions prepared from animal tissues observed that there was a rapid oxygen uptake by the system during the first five to 10 minutes, and then the rate fell



off sharply. The fall of the rate of oxygen uptake was so marked that at the end of one hour the final uptake was only slightly greater than at the end of the first few minutes. This effect was held to be due either to (a) the rapid destruction of the enzyme, or that (b) some product of the reaction inhibited the enzyme very strongly. The product of the reaction was assumed to be pyruvic acid and this assumption was confirmed by the fact that by the addition of cyanide to the system and thus by the formation of cyanhydrin, the accumulation of pyruvic acid was prevented. In the presence of an optimal concentration of cyanide the rate of the oxidation of lactate was increased markedly. The beneficial effect of cyanide was formulated as follows:



To confirm their assumption they added an excess of pyruvate and abolished the effect of cyanide; addition of a small amount of pyruvate had no effect. That the rapid fall of the oxygen uptake by lactate was due to pyruvate as reaction product was also demonstrated by adding 0.05 M pyruvate to the system at the beginning of the experiment in the absence of cyanide. This concentration of pyruvate completely inhibited the oxidation of lactic acid. While 0.04 M pyruvate inhibited the oxidation of l(+)-lactate 100 per cent, 0.03 M tartronate inhibited 60 per cent by virtue of configurational similarity to both the substrate and the reaction product.

The enzyme system studied by Green and Bostreaux shows a specificity similar to the high degree of serological specificity shown by antibodies against the optical, or d- and l-isomers of haptens coupled with proteins. Though it oxidizes l-lactate rapidly it has no effect on d-lactate, nor does a 0.03 M concentration of the latter exercise an inhibitory effect on the enzyme activity.

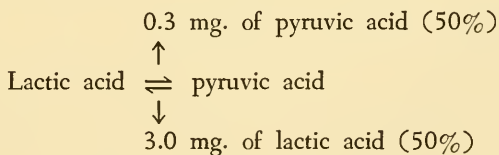
## 9. Inhibition of the Dehydrogenases of Succinic, Lactic and Malic Acids by Their Reaction Products

Das (1937a) investigated the affinity of lactic dehydrogenase towards both malic and lactic acids. The enzyme systems he used were

prepared from pigeon breast muscle, pig kidney and pig liver. He found that the "maximum concentration" (critical concentration)\* of lactic acid is about five to eight times greater than the maximum concentration of malic acid, thus showing that the affinity of the enzyme is much higher towards malic acid than towards lactic acid.

The question of whether the oxidation product of one substrate inhibits the oxidation of the other substrate was also studied: i.e., does oxalacetic acid, the oxidation product of malic acid, inhibit the dehydrogenation of lactic acid, and conversely does pyruvic acid, the oxidation product of lactic acid, inhibit the dehydrogenation of malic acid? In the former case the lactic acid dehydrogenation was inhibited to an extent of 50 to 60 per cent (by 1 mg. of oxalacetic acid), using the maximum concentration of lactic acid, while with the same concentration of oxalacetic acid the dehydrogenation of malic acid was also inhibited to the same extent. But pyruvic acid only inhibited the dehydrogenation of lactic acid and not the dehydrogenation of malic acid. This inability of pyruvic acid to inhibit the dehydrogenation of malic acid was explained by the fact that the affinity of the enzyme is much higher for malic acid than for lactic acid.

Das in a subsequent study (1937b) investigated the mechanism of inhibition of reversible dehydrogenation-hydrogenation systems by their respective reaction products. The experimental conditions were arranged so as to obtain 50 per cent inhibition. He found, for example, that the dehydrogenation of lactic acid to pyruvic acid was inhibited 50 per cent by 0.3 mg. of pyruvic acid. Conversely the hydrogenation of pyruvic acid to lactic acid was inhibited 50 per cent by 3.0 mg. of lactic acid. The reaction was presented as follows:



In the same way the dehydrogenation of malic acid to oxalacetic acid was inhibited 50 per cent by 0.02 mg. of oxalacetic acid, and the hydrogenation of the latter to malic acid was inhibited 50 per cent by

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\*"Maximum concentration" is the concentration at which the enzyme is most active.

3.5 mg. of malic acid. Similar effects were obtained in experiments on the reversible dehydrogenation of succinic acid to fumaric acid. Oxalacetic acid inhibited both the dehydrogenation of succinic acid and the hydrogenation of fumaric acid. Malonic acid inhibited the dehydrogenation of lactic, malic and succinic acids and the hydrogenation of their respective reaction products.

Das emphasized the point that the enzyme has greater affinity for the oxidized forms (i.e., pyruvic acid and oxalacetic acid) than for their reduced forms (i.e., lactic and malic acids). This was evident from the comparatively strong inhibition of the dehydrogenation of the reduced forms by pyruvic and oxalacetic acids.

## 10. Inhibition of Carboxylase by Acetaldehyde, the Decarboxylation Product of Pyruvic Acid

Since the discovery of carboxylase by Neuberg it has been variously reported that acetaldehyde resulting from the decarboxylation of pyruvic acid inhibits the activity of carboxylase. Lohmann and Schuster (1937) in their study on the isolation and properties of cocarboxylase determined the inhibitory effect of acetaldehyde in a system containing a yeast suspension, washed with alkaline phosphate, purified cocarboxylase and magnesium. During a 30 minute reaction period at optimal pH of 6.2 to 6.4 the inhibition of the decarboxylation of pyruvic acid by 2, 4 and 8 mg. acetaldehyde was respectively 25 to 30, 50 to 55 and 64 to 67 per cent.

## 11. Inhibition of the Oxidation of Purines by the Reaction Products of Purine Structure

There exists in milk and in most living tissues an enzyme capable of oxidizing the purine bases hypoxanthine and xanthine. Dixon and Thurlow (1924) studied this system in some detail. They used a preparation from milk. They reported that uric acid, a purine base, inhibited the oxidation of both hypoxanthine and xanthine, and the oxidation of hypoxanthine to xanthine. Other structurally related purine bases, such as guanine, adenine and xanthine itself were found to produce this inhibitory effect upon the oxidation of hypoxanthine.

The effect was, however, found to be remarkably specific for caffeine, but the pyrimidines uracil, cytosine and thymine showed no inhibitory effect. Histidine, which contains the imidazole ring, as in the purines, was also found not to have any effect. From the study of the kinetics of the reaction with hypoxanthine they concluded that the inhibitions of the oxidation of hypoxanthine by uric acid and by xanthine are essentially the same in nature.

Dixon (1926) in a later study determined the specificity of xanthine oxidase. This enzyme, with a high degree of specificity, oxidized only hypoxanthine and xanthine, and adenine to a slight extent, and most aldehydes. It had no action on (a) guanine, alloxan, quinoline, morphine, ricin; (b) tryptophane, ketones, benzylamine, peptone; (c) formate, acetate, lactate, succinate, citrate, glutamate; (d) caffeine, theobromine, uracil, thymine, cytosine, histidine, uric acid; and (e) glycine, tyrosine, alanine, serine, leucine and aspartic acid.

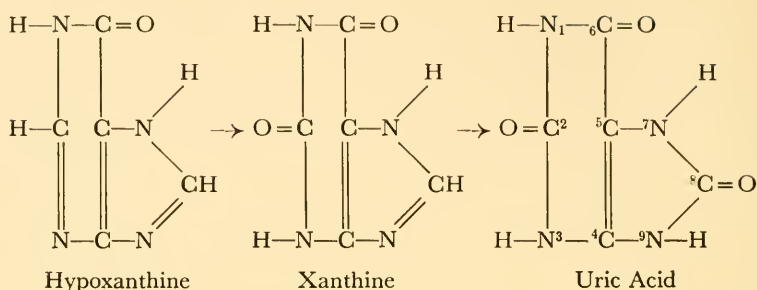
Coombs (1927) extending the studies on the specificity of xanthine oxidase found that the enzyme had no oxidizing action on 3-, 8-, and 9- methylxanthines, 1,3- and 3,8-dimethylxanthines, 1- and 7-methylguanine, 1,7-dimethylguanine and benziminazole. Beside hypoxanthine and xanthine 6,8-dihydroxypurine and 2-thioxanthine were readily oxidizable. The reaction products were uric and thiouric acids, respectively.

His studies further showed that the introduction of a single methyl group in either ring entirely prevented the oxidation.

In inhibition experiments hypoxanthine, xanthine, 3-methylxanthine, uric acid, adenine 6,8-dihydroxypurine, guanine, 1- and 7-methylguanine and 1,7-dimethylguanine were strongly adsorbed on the enzyme; 8- and 9-methylxanthine and alloxan adsorbed only to a small extent. Dimethyl- and trimethylxanthines and pyrimidines adsorbed only to a very slight extent. It is evident that the purine ring, and not the pyrimidine nor the imidazole ring, exhibits specific affinity for the enzyme.

The results of this study showed clearly that the degree of adsorption on, or the specific affinity for the enzyme was not only responsible for the oxidation of the purine bases, but also for the inhibition of the enzyme. The results also show that the simpler the structure of the purine compound, the greater is the degree of affinity exhibited for the enzyme.

## The oxidation of hypoxanthine

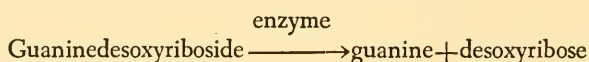


shows that the point of attack is at carbon 8 in the purine ring when xanthine is oxidized to uric acid and at carbon 2 when hypoxanthine is oxidized to xanthine. The fact that uric acid as a reaction product inhibits the oxidation of hypoxanthine and xanthine shows that it reacts with the same active grouping of the enzyme molecule which combines also with xanthine or hypoxanthine. As a result of this reaction the two xanthines become activated but uric acid not being activated by the enzyme can act only as a competitive inhibitor.

## 12. Inhibition of the Hydrolysis of Guaninedesoxyribose by the Reaction Product Guanine

Klein (1935) prepared an enzyme from spleen which was highly specific in hydrolyzing purine nucleosides. This enzyme preparation did not act on pyrimidine nucleosides, purine or pyrimidine nucleotides, or on d-ribose- or desoxyribose-nucleic acids.

To determine the specific affinity of nucleosidase for various substances he resorted to inhibition experiments, using guanine desoxyriboside as substrate.



The experimental procedure yielded 51 per cent hydrolysis of the nucleoside. Studying the effect of 18 different substances he found that the addition of 1 mg. each of guanine, hypoxanthine and adenine to the reaction system produced, respectively, 47, 53 and 21 per cent inhibition. Under identical conditions xanthine produced 10 per cent inhibition and uric acid had no effect whatsoever. It is evi-



dent that guanine as the reaction product of hydrolysis is a strong inhibitor of nucleosidase. While adenine exercises slight inhibitory effect, its deamination product, hypoxanthine, exercises greater inhibition. In contrast, the deamination of guanine to xanthine deprives the inhibitor of its effect on the enzyme. Five mg. of desoxyribose, one of the reaction products of hydrolysis, exercised only 10 per cent inhibition. Five mg. of each of the following substances: fructose, glucose, uracilriboside, cytosinedesoxyriboside, yeast and muscle adenylic acid, yeast and thymus nucleic acids, riboseguanilic acid were entirely ineffective.

### 13. Conclusion

In the preceding pages numerous experimental facts have been discussed showing how in reaction systems catalyzed by enzymes, substances are formed as final reaction products which specifically combine with the enzymes and inhibit their activity. This specific inhibition appears to compare with the inhibition of the biological activity of antigenic substances by antibodies which as final reaction products result from the catalytic action of antigens *in vivo*.

It would have been more expedient perhaps if we could have found examples from *synthetic* processes catalyzed by enzymes for a direct comparison with the production of antibodies resulting from the catalytic influence of antigens during the synthesis of globulins. As our knowledge of the mechanism of complex synthetic enzyme processes is very fragmentary, such a comparison was not possible. However, since as both processes are catalyzed, and in each case the final reaction products act as inhibitors on their respective enzyme systems, we believe the comparison is well within the limits of sound reasoning.

Our view concerning the synthesis and production of antibody as catalyzed by antigen differs from the theoretical basis of the experiments carried out by Pauling and Campbell (1942). We believe that antigen catalyzes and directs the synthesis of globulins from amino acids or polypeptides (see pp. 120, 156) in a specific direction to yield the homologous antibody globulin. Pauling and Campbell, on the other hand, have experimented with and advanced the idea that the complete globulin molecules can be converted into antibody molecules.

According to them the end chains of the globulin molecule can be uncoiled by a denaturing agent or condition, and during the process of recoiling, antigenic substances may intervene and impress their configurations on the recoiling end chains, yielding specific "antibodies." A similar experiment has been carried out by Bacon (1943) whereby whole plasma was dehydrated from the frozen state in the presence of an antigenic substance. The above investigators claimed to have obtained specifically reactive final "antibody" products. While none of these investigators have since produced additional corroborative results, as it was discussed earlier (page 116), various investigators have repeated these experiments and failed to confirm the above results.

## Part IV

# Anti-Enzyme Immunity

### A. ANALYSIS OF CERTAIN "CONTROVERSIAL" ASPECTS OF ANTI-ENZYME IMMUNITY

THE FORMATION and existence of anti-enzymes in animal systems or the production of antibodies against enzymes, by parenteral injection into animals of enzyme preparations, must be demonstrated by the same critical tests employed in toxin-antitoxin or other antigen-antibody reactions. The immune sera against enzymes must be specifically produced and must manifest specific serological properties or must specifically inhibit the activity of the homologous enzymes *in vitro* as well as in animals, whenever one or both of these tests are experimentally possible. If such anti-enzyme sera satisfy the known criteria of immune reactions, the antigenicity of enzyme proteins and, therefore, the existence of enzyme antibodies can be considered as an established fact.

The experimental data concerning anti-enzyme immunity have been divided into two categories to enable us to effect a reasonable comparison of both the enzyme and other immune processes, and the development of the various phases of the concept of "Immuno-catalysis," in a logical order.

We have already described in Part I of this treatise the preparation of numerous immune sera against crystalline enzymes which were tested by precipitation and anaphylactic reactions. The experimental data to be presented in this part of the treatise deal with the results obtained from experiments carried out in animals and *in vitro* with respect to the inhibition of the activities of enzymes by the homologous immune sera. However, before undertaking the presentation of the data certain controversial questions as to the existence of anti-enzymes must be analyzed in the light of available experimental facts.

## 1. Analysis of Bayliss' Objections Against the Existence of Anti-Enzymes

There have been published numerous studies in support of or against the existence of anti-enzyme immunity. Some of these studies date back to the last decade of the 19th and early part of the twentieth century. Numerous studies have been made and reported on this subject since. Bayliss' has been one of the chief antagonists of anti-enzyme immunity. One will find the list of his objections in the first and through the fourth edition of his *Principles of General Physiology* and also in all of the editions of his monograph on the *Nature of Enzyme Activity*. Since, through these publications, Bayliss has, perhaps, been very influential in shaping the reserve or perhaps the censorious attitude maintained by certain workers in the field of immunology regarding anti-enzyme immunity, it is necessary that his objections be analyzed. In his monograph on *The Nature of Enzyme Activity* Bayliss (1925) has presented the following four principal objections:

*First:* "The facts that enzymes are not proteins and that evidence is accumulating to show that their chemical constitution is of a simpler nature than was supposed at one time, are, *prima facie*, grounds for doubting their capacity of acting as antigens." We quote also the following paragraph from Wells' (1929) *Chemical Aspects of Immunity* which expresses practically the same view as the above, i.e., "At first both\* were believed to be proteins; now both are considered by many not to be proteins, but molecular complexes of nearly equally great dimensions."

The first objection of Bayliss, though the most important of the four, is the weakest. Studies carried out during the last twenty years have confirmed beyond doubt the older view of the protein nature of enzymes by isolating and subjecting to critical experiments numerous crystalline proteins with enzyme activities, such as urease, amylase, trypsin, pepsin, papain, d-ribonuclease, catalase and numerous other enzymes. These facts obviate Bayliss' principal objection.

*Second:* Discussing the merits of certain studies on anti-enzymes Bayliss claimed to have demonstrated that the absence of the proper

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\*Toxins and enzymes.

control of  $H^+$  ion concentration in these studies may have resulted in decreased enzyme activity, which fact was considered by him as responsible for an anti-enzyme effect. In the subsequent discussions of the experimental data published by various investigators, this question will be carefully analyzed and it will be shown that Bayliss' own data do not contradict but confirm the anti-enzyme effect as an immune reaction.

*Third:* "I have already pointed out that many of the 'anti' effects shown by serum can be accounted for by change of reaction, but this fact does not seem capable of explaining the increase of such effects stated to be produced by injection of enzymes. It is to be remembered, however, that when the normal blood already shows such properties, it is practically impossible to be certain that an increase following an injection is not due to a spontaneous change. Natural variations are, in fact, very considerable."

The implications of the above objection of Bayliss are vague. Since no specific experimental facts are mentioned in conjunction with the statement of this objection we refrain from analyzing it; we believe, however, that the experimental data to be discussed at length will show that the properties of anti-enzymes cannot be accounted for by ascribing them to non-immunological natural variations.

*Fourth:* "A further fact to be kept in mind is that substances capable of taking up enzymes by adsorption produce a diminution of their action merely by reducing their concentration." The examples he cited were: the adsorption of trypsin on charcoal; saponin prevents the adsorption and anti-action in a similar way to that in which it prevents the inactivation of rennet by shaking; the anti-tryptic action of serum has been shown to be associated with the albumin fraction of the proteins, not with the globulin fraction, as is usual with true antibodies. The probable interpretation of this fact, according to Bayliss, is "that the effect is due to an adsorption of the enzymes, this diminishing the effective concentration." He mentioned also the fact that when raw serum or egg-albumin is acted on by trypsin, it is found that no effect appears to be produced for some hours, and that gradually the enzyme begins to act and regains its power. This phenomenon is explained by him as follows: "The raw protein, for some reason not as yet clear, is difficult of attack, but adsorbs the enzyme. As it is slowly attacked and converted into products which have no ad-



sorbent properties, more and more of the enzyme is set free to act." He stated further: "There is no doubt of the existence of substances which have a markedly inhibiting action on certain enzymes, although it leads to confusion if they are called 'anti-enzyme,' since there is no evidence that they can be produced in response to the injection of these enzymes into organisms."

The question of non-specific adsorption of enzymes on various adsorbents as a possible cause of the diminished enzyme activity and of thereby accounting for the immune anti-enzyme inhibitory effect will be discussed under the heading of non-specific adsorptions. We will therefore discuss here the nature of the normal enzyme inhibitors found in the living cell and compare their properties with those of anti-enzyme antibodies.

**a. The Nature of the Trypsin Inhibitor Present in Sera in Relation to Anti-Enzyme Antibody.** The anti-tryptic action of certain normal sera, referred to by Bayliss, appears to us to correspond to the trypsin inhibitor of low molecular weight and of polypeptide nature which has been studied by Schmitz (1938). As discussed previously Schmitz isolated it from horse serum and characterized it as being similar to that crystallized from pancreatic extract by Kunitz and Northrop (1936). Both of these inhibitors are polypeptides of about 5000 molecular weight, which are not comparable at all with the anti-trypsin immune globulin described by Ten Broeck (1934). Antitrypsin antibody falls into the class of globulins having a molecular weight of about 150,000 to 160,000. It is also to be noted that the antitrypsin antibody is not present in the albumin fraction of the serum; in contrast, the trypsin inhibitor present in normal serum, as emphasized by Bayliss and demonstrated by Schmitz, is solely in the albumin fraction of serum. These facts make the further discussion of this subject unnecessary.

However, in this connection it is of interest to note an observation by Maschmann (1937). He found that the proteolytic activity of the toxin of *Cl. perfringens* was not inhibited at all by normal horse serum which was shown to exercise an inhibitory effect on other bacterial proteolytic enzymes. The proteolytic activity of the toxin, on the other hand, was strongly inhibited by antitoxic horse serum.

Smith and Lindsley (1939) studied the inhibition of the proteolytic enzymes of bacteria by immune sera. They separated the constitu-

ents of normal serum by electrophoresis in the Tiselius apparatus. The inhibitor was found primarily in the albumin fraction and the antiproteinase antibody in the globulin fraction.

Smith and Lindsley (1939) likewise found that while the trypsin inhibitor in normal serum had no inhibitory action on the proteinases of pathogenic *Cl. histolyticum*, *Cl. welchii* and *Cl. oedematis-maligni*, antiserum against the enzyme of *Cl. histolyticum* strongly inhibited the enzyme activity. Immune sera against the enzymes of the latter two organisms were not prepared.

**b. The Nature of the Trypsin Inhibitor in Egg White.** Balls and Swenson (1934; see also Balls and Hoover, 1940) isolated an inhibitor present in egg white; the purer form dialyzed slowly through collodion. It gave positive biuret and Millon tests, but was negative in a test with sodium nitroprusside, in a Molisch test and with Fehling's solution. A solution of 10 mg. per ml. gave no visible precipitate with picric, trichloroacetic, tannic acids, or mercuric chloride. With phosphotungstic acid, or with two volumes of saturated ammonium sulfate, a heavy precipitate formed. The total nitrogen content was 10.55 per cent; it was resistant to heat. These data show that the properties of the inhibitor are comparable to those of peptone-like substances, or to the trypsin inhibitor found in pancreas (Kunitz and Northrop) or in blood serum (Schmitz).

Balls and Swenson (1934) stated that they had corroborated the earlier findings of Delezenne and Pozerski (1903) that this inhibitor combines with kinase, because additional amounts of kinase decrease the inhibition. Furthermore, they found that the reversal of inhibition by kinase becomes more marked as the amount of inhibitor is decreased. On the other hand, additional amounts of inactive enzyme (trypsinogen or protrypsin) have also the effect of removing the inhibition. The inhibition is therefore a reversible reaction.

The interpretation of the above mentioned reactions was based on the then current idea that "trypsin-kinase" was a definite compound. Since the publication of the above paper, various enzyme components of pancreatic extracts have been obtained in crystalline form and studied for their interactions (Kunitz, 1939; Kunitz and Northrop, 1936). The results of these studies may be compared to those obtained by Balls and Swenson with the inhibitor from egg white.

According to Kunitz and Northrop (1936) and Northrop (1939)

enterokinase activates crude chymotrypsinogen to chymotrypsin. In contrast, crystalline trypsin fails to effect this activation. This failure was attributed to the presence of a trypsin inhibitor in the crude chymotrypsinogen which combines with trypsin and inhibits its activating property. After one crystallization of the crude chymotrypsinogen, however, the inhibitor remains in the mother liquor; trypsin then is capable of activating chymotrypsinogen to chymotrypsin.

The crude chymotrypsinogen also contains trypsinogen, which, acted upon in the same manner (removal of inhibitor) by enterokinase, is transformed into trypsin. A sufficient amount of active trypsin thus formed is capable of overcoming the inhibitory action of the solution, resulting in the activation of chymotrypsinogen. The same result is obtained by adding enough trypsin even in the presence of the inhibitor.

Balls and Swenson (1934) and Balls (personal communication) stated that the trypsin-inhibitor of egg white was incapable of inhibiting the activity of papain. One positive effect on papain was attributed to something else, since the search for the same positive effect with other papain preparations utterly failed. Ross and Tracy (1942) have studied the effect of the inhibitor of egg white on the digestion of casein by chymotrypsin. They observed only 15 to 20 per cent inhibition. However, if the inhibitor was first incubated with casein (at 37.5°C. for 40 minutes) before adding the enzyme, no inhibitory effect was observed.

The delayed action of trypsin on raw egg white described by Bayliss could not be due to this inhibitor for the following reasons. The trypsin used by him was capable of hydrolyzing heat denatured egg white. Since the inhibitor as found by Balls and Swenson is resistant to heat, it should be still present in the heat denatured egg white in an active form and therefore inhibit the active trypsin. Since heat denatured egg white exercised no inhibitory effect on trypsin, the delayed action of trypsin on raw egg white must be due to some other factor. The possible nature of this factor is discussed below.

**c. The Resistance of "Living" Protein to the Action of Proteolytic Enzymes.** It has been known for a long time that all living cells are resistant to proteolytic enzymes. Dead organisms are rapidly digested by them. Several hypotheses have been advanced to explain this fact.

Among others, the following may be mentioned: The presence of enzyme inhibitors (or anti-enzymes) in the living cells; the presence of passive or active protective membranes; selective impermeability of the cell membranes to the proteolytic enzymes; the existence of repulsive forces between the cell membrane and the proteolytic enzymes, etc. After a critical analysis of these hypotheses Fermi (1910) rejected them and arrived at the conclusion that the resistance of "living" proteins is due to a difference of "biochemical constitution" between the proteins of living and dead cells. Northrop (1926, 1939) stated that he confirmed the conclusion of Fermi. Of the above hypotheses, the impermeability of the living and the permeability of the dead cells to proteolytic enzymes appears to have been one of the most important aspects. Northrop stated that "in every case that as long as the cell was alive, no detectable quantity of enzyme was taken up; whereas when the cell dies, the enzyme was rapidly removed from solution and concentrated in the cell."

In the opinion of Northrop the digestion of heat-killed organisms may be accounted for by assuming a change in the chemical nature of the proteins, or the destruction of the anti-enzymes. These objections cannot, however, account for the digestion of the organisms (earthworm and mealworm) when killed simply by mechanical injury. It is known that there is an inhibitor in organisms such as the earthworm (*Lumbricus terrestris*). The presence of this inhibitor might be considered as the reason for the resistance of the living earthworm to the action of trypsin. This can, however, be ruled out in view of the fact that in the presence of an amount of trypsin in excess of the amount required to neutralize this inhibitor the tissue of the earthworm killed by cutting is found to be digestible (Northrop, 1926).

In the light of what has been said above, the delayed action of trypsin or raw egg albumin in Bayliss' experiment does not appear to be due to an inactivation by adsorption. The gradual "reversal of activity" beginning with the tenth hour of the reaction period, as described by Bayliss, and that at the end of the 70th hour, the activity of trypsin on raw egg albumin and on egg albumin denatured at 100°C. was equal, may indicate a gradual denaturation of raw egg white by dilution with nine volumes of water and standing in contact with the reaction system for such a long time. The denaturation of



egg white with relative ease would appear to account for the facts described by Bayliss.\*

The ability of trypsin to digest the type specific M protein from living streptococci is reported by Lancefield (1941). She stated that the M substance in the living bacteria is readily accessible to the action of proteolytic enzymes without injury to other vital functions (viability, virulence and the ability of the multiplying streptococci to synthesize M) of the living cells. This, she concluded, may be due to its possible location near the outer surface of the streptococci.

M is an unsymmetrical protein of about 41,000 molecular weight (Pappenheimer, *et al.* 1942). The ratio of major to minor axis is at least 20 to 1, which puts it into the class of "denatured" or unfolded proteins. Since the process of extraction from streptococci (16 hours at 56°C. with 0.05 N HCl containing 2 per cent sodium chloride) and subsequent treatments with alcohol, etc. do not affect the serological type specificity of M, it is possible that the constitution of this protein in its natural environment may be similar to that of the isolated form. These facts may perhaps explain why M, of all the other cellular proteins, is apparently selectively digested by trypsin.

Impermeability of living cells to proteolytic enzymes as the cause of the resistance of "living" proteins to these enzymes does not account for the resistance of extracellular native proteins to proteolytic digestion. Anson and Mirsky (1933), and Anson (1938) reported that hemoglobin denatured by salicylate is digested by trypsin which does not attack native hemoglobin. The denatured hemoglobin was insoluble under the same conditions under which native hemoglobin was soluble; it had the parahematin type of spectrum which is also given by a solution of hemin in pyridine. When the denaturation of hemoglobin by salicylate is reversed, the original properties of native hemoglobin are restored.†

Bawden and Pirie (1937) also found that crystalline tobacco mosaic virus is resistant to proteolytic enzymes. They stated that no enzyme

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\*In this connection an observation by Pozerski and Guelin (1938a) is of importance. They found that raw egg white exercised no inhibitory effect on the gelatinase activity of *B. histolyticum*. In contrast the proteolytic activity of this organism was strongly inhibited by immune horse serum against *B. histolyticum*. This serum exercised no inhibitory action on the proteolytic activity of closely related *B. sporogenes*.

†In this connection it is to be noted that active bacteriophage and botulin toxin similar to native hemoglobin, are resistant to proteolytic enzymes (Kalmanson and Bronfenbrenner, 1943).



preparation has yet been found that attacks purified virus preparations at an appreciable rate, or that has any permanent effect on their infectivity. They tried the proteolytic effect of trypsin, pepsin, papain and autolyzed preparations of kidney at a number of pH values around that optimal for enzymic activity, but in no case were they able to observe any proteolytic activity on the living virus. In contrast, all these enzymes were shown to be strongly active proteolytically when tested against the heat denatured virus which was rapidly hydrolyzed. In the presence of a large amount of trypsin the infectivity of the purified virus preparations was reversibly inactivated as a result of a possible virus-trypsin complex formation. This inactivation of the infectivity was observed to occur immediately after the virus and enzyme were mixed, and no further loss followed incubation. By precipitation with acid or dilute ammonium sulfate solution the virus was recovered with its full activity from such non-infective mixtures. Similarly, when various amounts of a solution of papain were added to constant amounts of virus, a papain-virus precipitate was obtained. From these precipitates the active enzyme was recovered by extraction at pH 3.3. Virus was also recovered without undergoing any change in chemical, infective or serological properties.

According to Kleczowski (1944) pepsin combines with virus X (without affecting infectivity, suggesting that different parts of the virus particles are involved in combination) and casein, which are substrates for its proteolytic activity, but not with tobacco mosaic virus, which is not a substrate. Tobacco mosaic virus denatured by heat is readily hydrolyzed by pepsin and combines with pepsin almost to the same extent as potato virus X. On the other hand, more trypsin combined with tobacco mosaic virus (with loss of infectivity), which is not a substrate for its proteolytic activity, than with potato virus X, which is a substrate. The combination of trypsin with tobacco mosaic virus could account for the reversible inhibition of infectivity of the virus by trypsin. This combination protects trypsin from spontaneous inactivation at pH 7.0.

Invertase does not combine with potato virus X, with tobacco mosaic virus, whether heat denatured or not, or with casein.

d. **The Inhibition of Enzymes by Enzymes and Viruses.** Bayliss emphasized also the presence of normal enzyme inhibitors in living cells, particularly a proteolytic enzyme inhibitor present in the worm

*ascaris*. Weinland (1903) isolated a substance from *ascaris* capable of inhibiting both peptic and tryptic activity. He concluded that the inhibitor acted by combining with the enzymes and that its function was to prevent the destruction of the parasite by the host's digestive juices. In the light of the findings of Fermi, Northrop and others, discussed above, that "living" cells or living protein molecules are not attacked by proteolytic enzymes, Weinland's conclusions do not appear to be acceptable. Furthermore Sang's (1938) findings throw a different light on the subject. Though Sang's experimental findings corroborate the principal part of Weinland's findings, his data suggest that the anti-peptic and antitryptic substance of *ascaris* is a protease.\* He extracted it by grinding the cut up worms to a mass in a mortar and extracted the ground mass with water for 3 days under sterile conditions. The extract filtered through a Berkefeld filter was found to be stable on boiling in acid solution. In fact the proteolytic activity of the preparation increased by coagulating and removing the extraneous proteins. On boiling from five to 36 hours the activity was lost with the disappearance of the biuret reaction. It was completely precipitable by less than half saturation with ammonium sulfate. This, with its ready diffusibility through parchment and its precipitability by 70 to 80 per cent alcohol made it probable that this substance was an enzyme of small molecular weight or was associated with a substance of the order of a primary albuminose. Sang called this substance *ascarase*.

This preparation exercised proteolytic activity on casein and pepsin in buffered reaction systems at an optimum pH of 5 to 7. Since it was also found that the maximum inhibition of trypsin by this substance lay within the same pH range, Sang held it probable that the two enzymes combine with each other at a pH range of their maximum activity as they would with their normal substrates. This combination between the two enzymes was rapidly formed and easily reversible. It was found that *ascarase* was partly destroyed by standing in solution at neutrality, while in its combination with trypsin it was not destroyed. The study of the kinetics of numerous reaction systems revealed that

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\*According to Harned and Nash (1932) extracts of *Ascaris lumbricoides* contain both a trypsin inhibitor and a proteolytic enzyme of optimal activity at pH 8 and 37.5°C. They found that this trypsin inhibitor (free from the worm proteinase) protected isoelectric insulin at pH 8 and 37.5°C. against the proteolytic action of strong pancreatic solutions.

the inhibitory and the proteolytic powers of *ascarase* ran parallel. It inhibited trypsin and pepsin to the same degree, but had no effect on papain.

Northrop (1933) found that crystalline edestin adsorbs pepsin from a solution, removing it completely at pH 4.0. The pepsin content of "edestin-pepsin" complex was found to be as high as 50 per cent. In this form the pepsin activity was not arrested. A suspension of "edestin-pepsin" complex on standing at room temperature gradually dissolved, eventually completely, so that the final solution consisted of the digested edestin containing the original quantity of pepsin.

A combination between crystalline d-ribonuclease and tobacco mosaic virus resulting in the reversible inactivation of the virus was reported by Loring (1942). A virus-enzyme complex formed long fibre-like particles, which on analysis proved to contain about 14 per cent d-ribonuclease. This complex was dissociable completely, liberating the virus in fully active form. The dissociation took place by diluting the solution of the complex from 1 to 500 to 1 to 1000 times. The liberation of virus from the virus-enzyme complex was achieved also by sedimenting it at high speed and redissolving the pellets. On repeating this process a few times 92 per cent of the virus was recovered.

## 2. The Question of Non-Specific Adsorption of Toxins or Enzymes on Coexisting Protein-Anti-Protein Precipitates

The diminution of the activity of enzymes in the presence of homologous immune sera was assumed by Bayliss to be due also to non-specific adsorptions on extraneous protein impurities in the form of antigen-antibody precipitates. This question of non-specific adsorption as the cause of serological reactions has often been raised since the early beginnings of the science of immunology and has been critically considered in numerous studies. However, since there still seems to be an element of doubt in the mind of many workers regarding the existence of immune anti-enzymes, it is necessary that the available data regarding this question be further analyzed.

At first a few examples of adsorptions of enzymes on materials, such as were mentioned by Bayliss, will be given. There is no doubt

that enzymes adsorb on charcoal, alumina, Fuller's earth, etc. in a manner similar to the adsorption of toxins on these adsorbents. However, one very seldom carries out immune reactions in the presence of such adsorbents; secondly, it does not necessarily lead to the inactivation of the enzymes or toxins. The decrease of the activity or absence of inactivation in the adsorbed state depends on what particular groups of the enzymes or toxins have been blocked by the adsorption process.

Griffin and Nelson (1916) found that invertase adsorbed on aluminum hydroxide or on small amounts of charcoal is just as active enzymatically as the solution of free enzyme. The presence of egg-albumin in invertase solution likewise does not affect the enzyme activity. Michaelis (1921) reported that invertase adsorbed on Fuller's earth or iron oxide, likewise, is just as active as the free enzyme. Fructose, mannose, lactose,  $\alpha$ - and  $\beta$ -methylglucoside fail to elute the enzyme from the adsorbent. Sucrose and raffinose which are hydrolyzed, and maltose which is not hydrolyzable by invertase, slowly promote the elution of the enzyme. These facts show that the elution effects of the former two substances are not related to their being substrates for the enzyme. On the other hand, those substances, glucose, fructose, mannose,  $\alpha$ -methylglucoside, which show a great affinity for and thereby inhibit invertase activity, fail to effect the elution of the enzyme.

According to Kleczowski (1944) invertase adsorbed on charcoal can be set free by casein, and it can be extracted by tobacco mosaic virus, but not by sucrose.

Oparin and Kurssanow (1929) precipitated invertase by tannic acid; in this state invertase was inactive. It is interesting to note that by shaking the tannic acid precipitate of the invertase preparation with egg albumin or peptone they stated they had formed egg albumin-tannate, or peptone-tannate precipitates, setting free the invertase in an active form. Evidently egg-albumin-tannate, or peptone-tannate precipitates are incapable of adsorbing the liberated invertase.

Freund (1931) reported that tannin detoxifies diphtheria and tetanus toxins in solution or adsorbed on collodion particles. This effect operates *in vitro* as well as *in vivo*. Tannin combining with the toxins produces a precipitate. The degree of detoxification runs parallel with the amount of precipitate formed. However, this effect is reversible at pH 8, or in high dilutions, and the toxin is recovered in an intact



form. Klopstock and Neter (1933) reported that tannin detoxifies *cobra* venom and ricin *in vivo*, as well as their hemolytic effect *in vitro*. However, these inhibitions by tannin were reversible by simple dilution of the detoxified reaction mixtures. It is apparent from these studies that the inhibitory action of such substances is easily reversible, and has nothing in common with the inhibitions brought about by immune bodies.

Lüers and Albrecht (1926) in a study on immune anti-amylase, investigated this particular question of non-specific adsorption of an enzyme on a coexisting antigen-antibody precipitate to meet this specific objection of Bayliss. The amylase activity in the presence of egg albumin-anti-egg-albumin precipitate showed no decrease.

Since data of sufficient scope regarding the non-specific adsorbability of enzymes in an antigen-antibody reaction environment are not available, we will discuss as a means of comparison the data regarding the non-specific adsorbability of toxins on serological precipitates. Such a comparison between toxins and enzymes is justified on the basis of the resemblances of their physico-chemical properties, as well as the enzymic properties manifested by certain toxins. These resemblances have been observed for many years (Wells, 1929). The same difficulty is encountered in isolating toxins and enzymes; both are approximately of similar molecular dimensions and are non-dialyzable through animal or other membranes; they pass through porcelain filters; they possess similar adsorption properties; neither will stand boiling and most forms are destroyed at 80° instantly or in a short time; left standing in solution for some time they gradually lose their specific properties, the toxin becoming toxoid and the enzyme a fermentoid.

Besides the above physico-chemical resemblances between the toxins and enzymes they exercise two other properties which bring them closer. The first is the high degree of activity of toxins which compare favorably with those of enzymes. The second is the fact that toxins often show enzyme activities, whereas certain enzymes show toxic activities. Eaton (1936) found that tetanus toxin of certain purity is fatal in a quantity of 0.4 microgram per kgm. body weight of a guinea pig. Sommer (1937) found that 0.2 microgram of botulinus toxin per kgm. of the body weight of mouse is fatal. Eaton (1936) and Pappenheimer (1937) found that 0.4 microgram of diphtheria toxin per kgm. body weight of guinea pig is fatal.



As will be discussed in detail in the latter part of this study, the toxic effects of several toxins are correlated with their proteolytic or lecithinase activity. On this and on the grounds cited above, a comparison of the adsorbability of toxins with that of enzymes appears to be justified.

a. **Adsorption of Toxins on Red Blood Cells and Charcoal without Loss of Activity.** Sbarsky (1923), and Sbarsky and Michlin (1923) stated they had found that inactivated diphtheria toxin adsorbs on red blood cells *in vivo* and *in vitro*, and that there is a relationship between the adsorption property of the cells and the susceptibility of the animal to diphtheria. They found that diphtheria toxin is adsorbed by the red blood cells of various animals in the following order of adsorptive power expressed in percentages: rat, 14; rabbit, 75.8; horse, 88; guinea pig, 91.8; hen, 93; pigeon, 95.

The above observation appears to have been corroborated by the findings of Dujarric de La Rivière and Kossowitch (1932). They stated that red blood corpuscles adsorb diphtheria toxin and that the adsorbing ability of the red blood cells varies among different animals. The blood plasma did not fix, or it fixed only a very small amount of toxin. Levine (1939) was able to remove staphylococcal exotoxin quantitatively by adsorbing on red blood cells.

Eisler (1922) studied the reactivity of toxins and antitoxins after adsorbing them on charcoal. The activity of vibrio toxin adsorbed on charcoal was not altered. In this state, it was capable of combining with an equivalent amount of neutralizing antitoxin. In contrast, vibrio toxin adsorbed on red blood cells was incapable of combining with antitoxin. Diphtheria and tetanus toxin adsorbed on charcoal manifested weaker combining properties for the respective homologous antitoxins. For this reason, they required greater amounts of antitoxin for neutralization. Eisler correlated this behavior of tetanus or diphtheria toxin adsorbed on charcoal with the fact that in serum treatment they require a larger amount of antitoxin than the equivalent amount. Despite the weakening of the combining properties of charcoal adsorbed toxins, their injurious effect on tissue was not diminished or altered.

b. **Failure of Protein-Anti-Protein Precipitates to Adsorb Toxins.** Maloney and Weld (1925) investigated the neutralization of toxin with specific antitoxin in the presence of other bacterial impurities

and their antibodies. Horses were immunized against diphtheria culture fluid filtered through a Berkefeld candle. The immune horse sera contained agglutinins against the diphtheria bacilli. A mixture of diphtheria agglutinating sera (which contained no antitoxin) and crude toxin gave a precipitate. After centrifuging the reaction mixture the supernatant showed a marked drop in agglutinin content. This same supernatant showed no measurable fall in the toxin content determined by skin tests on guinea pigs. Conversely, a plasma containing both agglutinin and antitoxin after flocculation of antitoxin with toxin showed no change in the agglutinin titer of the centrifuged supernatant. Schmidt (1926) carried out similar tests as described above. After treating a diphtheria agglutinating serum with crude toxin and removing the floccules by centrifuging, he compared the toxicity of the supernatant with a control sample of toxin treated with saline and found no difference in their strength. These facts show that the protein impurity present in the toxin solution reacts with its homologous antibody, and that the resulting precipitate does not adsorb detectable amounts of the toxin present in the mixture. In an agglutinating serum which also contains antitoxin the two reactions, toxin-antitoxin and agglutinin-agglutinin, take place independently.

Marrack and Smith (1931) tested the mutual adsorbability of an azo-globulin dye solution (prepared by linking purified horse serum pseudo-globulin, or crystalline egg-albumin with diazotized atoxyl) with diphtheria toxin-antitoxin floccules. Diphtheria toxin was mixed with azo-globulin solution and then an equivalent amount of antitoxin was added. The mixture was kept for three hours at 45°C. and overnight in the ice-chest. The centrifuged precipitate, dissolved in 0.01 N NaOH solution, was completely colorless. No azo-globulin was therefore carried down with the toxin-antitoxin floccules.

**c. Failure of Protein-Anti-Protein Precipitates to Adsorb Non-Specific Colored Proteins.** Marrack and Smith (1931a) mixed anti-pseudoglobulin serum with azo-egg albumin solution; then one-third the optimum proportion of horse pseudo-globulin was added. The mixture was kept five hours at room temperature and in the ice-chest overnight. The precipitate on dissolving in 0.01 N NaOH solution was completely colorless. No dye azo-albumin was therefore carried down by the pseudoglobulin-anti-pseudoglobulin precipitate. The absence of azo-protein in the precipitate was determined spectroscopically with

ultraviolet radiation of 3600 Å. At this wave length only azo-protein was measured. Over the range of concentrations measured Beer's law was found to be obeyed whether other proteins were present or not (see further, Smith and Marrack, 1930; Marrack and Smith, 1931b; Marrack, 1938b).

Heidelberger and Landsteiner (1923) in a study on the specificity of hemoglobins obtained from various species of animals, compared the color of precipitates produced in the following combinations:

- (1) Hemoglobin + anti-hemoglobin serum = red precipitate;
- (2) Hemoglobin + horse serum + anti-horse serum = pure white precipitate;
- (3) Hemoglobin + human serum + anti-human serum = pure white precipitate;
- (4) Hemoglobin + donkey serum + anti-donkey serum = pure white precipitate;

The absence of any color (hemoglobin) in the precipitates of (2) to (4) combinations demonstrated clearly that hemoglobin was not dragged down non-specifically by these three different serum-anti-serum precipitates.

Haurowitz and Breinl (1933) precipitated 400 ml. of 1:1000 normal horse serum with 40 ml. of anti-horse rabbit serum in the presence of colored beef atoxyl-albumin containing 8.24 mg. of protein with a 61.2 microgram arsenic content. After 24 hours the precipitate was centrifuged and washed once with saline. The precipitate was pure white and free from dye. The dry weight of the precipitate was 55.2 mg. It contained less than 0.1 microgram of arsenic or at the most 0.03 per cent atoxyl-protein. Ten ml. of human serum (1:100 dilution) was treated with atoxyl-horse globulin, containing 3.0 mg. of protein with 16.7 microgram of arsenic, and treated with 1 ml. of anti-human serum. The precipitate was pure white and free from arsenic.

**d. Failure of Protein-Anti-Protein Precipitates to Adsorb Non-Specific Proteins.** Heidelberger and Kendall (1935) in their quantitative studies on the mechanism of precipitation reaction subjected the question of the effect of non-specific proteins on the amount of antigen-antibody precipitates to a critical study. They found that at 0° or 37° the ratio of nitrogen to Type III pneumococcus polysaccharide in the precipitate was of the same order whether the precipitation was carried out in the whole serum in which the antibody constituted about 15 per cent of the total protein, or in the antibody solution prepared

from it, in which antibody was 50 to 60 per cent of the total protein. In other parallel experiments, carbohydrate precipitation determinations were studied with antibody solution alone, and with antibody solution to which an equal volume of normal horse serum had been added. The non-specific protein had no effect upon the amount of antibody nitrogen precipitated under any conditions of temperature investigated.

**e. Failure of Agglutinated Bacteria to Adsorb Non-Specific Proteins.** Heidelberger and Kabat (1934, 1936, 1937) investigated the question as to whether non-specific proteins were adsorbed on bacteria in an agglutinating system. They found that the amount of antibody nitrogen taken out by the bacterial after 48 hours at 0°, with occasional stirring, was independent of the volume just as in the precipitation reaction. Thus the antibody nitrogen removed by agglutination was independent of the concentration of antibody nitrogen in the supernatant.

Pneumococcal cells (Type I), agglutinated with a considerable excess of antiserum, were washed with saline until the supernatant contained no agglutinin. The cells agglutinated in the region of excess antibody, they believed, would still have available on the surface of the particles some of the specifically reactive groupings of the originally multivalent antibody. These particles, then, should be able to combine with Type I pneumococcal carbohydrate on the surface of freshly added unsensitized Type I pneumococcal cells, and reagglutination should take place to form larger aggregates. This assumption was verified and the effect was found to be specific, since it was not given by pneumococcal Types II or III, or by Type I R cells under identical conditions of salt concentration. Reagglutination was, moreover, produced almost as completely by suitable amounts of Type I carbohydrate in solution, so that the conclusion appeared inescapable that these particulations, as well as the original antigen-antibody combination, were a chemical, and not a non-specific adsorption process.

While the finely and uniformly resuspended pneumococcus-agglutinin complex was found to reagglutinate completely with the addition of 0.01 mg. and partially with 0.0001 mg. of Type I pneumococcal polysaccharide, no visible reaction was observed with 0.1 mg. of Type II pneumococcal polysaccharide. When the upper fluid containing the Type II cells in suspension was decanted away from the mass of the Type I pneumococcus-antibody agglutinate, and the decanted suspen-



sion (containing Type II cells) was treated with Type II antiserum agglutination took place.

## Conclusion

In the preceding pages certain controversial aspects of the question of the existence of anti-enzymes were analyzed. The points considered no doubt apply primarily to impure enzyme preparations. In the light of the above cited facts showing the absence of non-specific adsorption of proteins on antigen-antibody precipitates,\* the objections in this regard appear to have been merely of a speculative nature. Furthermore, in studies in which crystalline or highly purified enzymes have been used these objections are stripped of their power and are only of historical interest.

## 3. Effect of pH of Optimal Enzyme Activity on the Nature and Extent of the Antigen-Antibody or Enzyme-Anti-Enzyme Combinations

Each enzyme exercises its greatest activity at a narrow range of pH. Pepsin, for example, is most active at pH 2 to 3. In contrast trypsin and papain function best at neutrality.  $\beta$ -Amylase is most active at pH 5.2;  $\beta$ -fructosidase (invertase), pH 4.5;  $\alpha$ -glucosidase, pH 7.5 to 6.5; in contrast,  $\beta$ -glucosidase functions best at pH 3 to 6. The pH of the optimal activity of bacterial enzymes varies. The cytochrome-cytochrome oxidase systems function at neutrality; on the other hand, carboxylases are most active at or around pH 6. In the study of the nature of the combination between any one particular enzyme and its homologous antibody it might appear necessary to work in a region of acidity where the enzyme is most active. However, the pH of optimal enzyme activity might not be the favorable one for complete enzyme-antienzyme combination. It is necessary that the test be carried out at a pH favorable for immune reactions. As a direct result of the chosen pH the activity of the enzyme might be less than the optimum. This, however, is unavoidable if we are interested in demonstrating the inhibition of the activity of an enzyme by its homologous antibody.

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\*This statement does not apply to the combination between the antigen-antibody complex and the proteins comprised in complement. In complement fixation the complement combines non-specifically with various antigen-antibody complexes.



Schubert (1933), in a study on the retardation of invertase activity by homologous immune sera, found that from pH 3 to pH 4.2, normal and immune sera produce the same effect, the inhibiting property of immune serum vanishing. The presence of either normal or immune serum in this acid region enables the invertase to retain 90 to 92 per cent of its activity at pH 4.5 to pH 5. From pH 5 on, on the alkaline side, invertase activity is not altered in the presence of normal serum. In contrast, the retardation by immune serum between the range of pH 4.5 to pH 6.5, respectively, rose from 0 to 25.6 per cent. (At pH 6.5 the activity of invertase *per se* was 50 per cent of its activity at its optimum pH of 4.5.) These findings showed that in the acid region the combination between the invertase and anti-invertase was completely prevented, and that within a range of pH 5 to 6.5 union took place producing retardation of the invertase activity.

In view of these facts, a review of the literature regarding the effect of pH on antigen-antibody combination is necessary. Mason (1922) stated that precipitation reactions occurred between pH 4.5 and 9.5 and outside this range, immune precipitates dissolved. Schmidt (1930) found that diphtheria toxin and antitoxin flocculated rapidly at pH 5.0 to pH 8; outside of this range the toxin is partly destroyed and the flocculation is slow or does not take place.

Bayne-Jones (1924), studying the effect of pH 4.5, 6, 8 and 9 on the rate of toxin-antitoxin flocculation, stated that the results of titrations at pH values beyond the range of 6.4 to 8.4 were irregular and not significant. When the toxic broth was made more acid or alkaline than pH 6.4 to 8.4 non-specific precipitates were produced in the broth, which obscured any flocculation due to the union of toxin and antitoxin.

Brown (1934), in a study of the effect of pH, ranging from 8.0 to 4.77, on the optimal flocculation values, stated that the greater the pH, the more pneumococcal Type I and Type II polysaccharide is necessary to combine with the antibody. Appreciable effect both on the amount and speed of flocculation was noted below pH 6.26. The pH effects were very similar to those of increasing salt concentration; that is, the more salt in the flocculation mixture, the more antibody was necessary to form a stable compound with the polysaccharide.

Marrack and Smith (1930, 1931) stated that they observed no effect at pH values from 8.0 to 6.6 on the ratio of antibody to antigen in the

precipitates. Heidelberger and Kendall (1935) in their study on the quantitative theory of the precipitin reaction found no difference, within the range of pH 6.7 to 7.9, on the weight of antigen-antibody precipitates. These investigators made no mention of the effect of lower pH values.

Pressman, *et al.* (1948) reported that the precipitation of p-azosuccinylate ovalbumin antiserum with homologous antigen, and that of the protein antigen of antiserum specific to the p-azophenylarsonate ion and the p-(p-azophenyl)-phenylarsonate ion is optimum at pH values of 7.4 and 8.1. This range of pH is also optimal for the precipitation of other azoprotein antigens with negatively charged haptenic groups.

Optimal pH for complete agglutination of sensitized cells has been found to correspond with the iso-electric pH of the antibody globulin. At the isoelectric pH of the antibody globulin, the largest amount of sensitizing antibody is found to be absorbed by the cells (Coulter, 1920; DeKruif and Northrop, 1922-1923; Joffe, 1935).

It is apparent from these studies that the most favorable pH for complete antigen-antibody combination is the region near to neutrality. It is desirable, however, that in an investigation of enzyme-anti-enzyme reactions, this aspect of the question be carefully studied. As an interesting illustration of the importance of pH on immune reactions the findings of Northrop (1932) from a study of the serological behavior of pepsin are described here. He immunized rabbits using intraperitoneal injections with either active or inactive pepsin preparations. The immune serum against active pepsin gave a precipitate with active pepsin in a dilution of about 1:2000, and with inactivated pepsin in a dilution of 1:16. The serum against alkali inactivated pepsin gave a precipitate with both the inactivated and active pepsin in a dilution of about 1:8.

The inhibition of the activity of pepsin (tested with a casein solution) by either of the immune sera was about the same and not very much greater than the inhibition by normal serum. The inhibitory effect, measured by the rate of hydrolysis of gelatin with a small amount of pepsin to which increasing amounts of the various sera were added, was about 40 per cent with serum against active pepsin in a dilution of 1:4, and about 20 per cent with denatured pepsin serum, and still less with normal serum. The weak inhibitory effect

of antipepsin immune serum was explained by Northrop by the fact that the active pepsin injected must have been almost instantly inactivated in the animal body, due to the neutral reaction of the circulating body fluids. For it was shown that pepsin is instantly more than half inactivated at pH 6. It would therefore seem that it is almost impossible to prevent the denaturation of active pepsin in the animal system; antibody may be formed against the denatured enzyme.

## B. A CRITICAL CONSIDERATION OF THE ANTIGEN-ANTI-BODY REACTIONS IN RELATION TO THE INHIBITION OF ENZYMES BY SPECIFIC ANTIBODIES

### 1. An Analysis of the Reactions of the Active Groups of Proteins in Relation to their Biological Specificities

The ability of enzyme proteins to stimulate the formation of specific antibodies is now an established fact. There are, however, questions pertaining to the inhibition of the enzymes by respective antibodies which require consideration. The complexity of these questions and the inadequacy of the available data do not permit us at present to treat them satisfactorily. One can only raise questions and discuss them as plausibly as possible. These questions may be formulated in the following manner:

(1) Are the SH, -S-S-,  $\text{NH}_2$ , tyrosine, etc., groups which are studied in relation with the inactivation or activation of certain enzyme proteins the sole determinant factors? Or are the reactions involving these groups simply superficial manifestations of other more significant changes which the protein molecule undergoes which we have not as yet been able to determine.

(2) Do the anti-enzyme antibody molecules contain specific combining sites elicited in response to the stimulation of the active groups of enzymes?

(3) Do the inhibitions of enzymes by homologous antibodies result directly from the interaction of the respective specifically reactive groups of enzymes and antibodies in a manner identical with those of other antigens and antibodies? Or are these inhibitions due to secondary effects resulting from the formation of antigen-antibody complexes?

(4) Are the inhibitions due to the mechanical blocking of the en-

counter of the substrates with the enzymes? That is, does the combination of the antibody with the enzyme block, without combining with, the active areas of the enzyme preventing the contact between the substrate and these areas?

**a. Studies on the Reactive Groups of Proteins.** In recent years, certain phases of the first question have been studied and continue to be a lively subject of interest. Let us briefly discuss the results of such investigations. Investigators have pursued the line of reasoning that, if the biological activity of a purified protein is first lost by the action of group-specific reagents, and then, after reversal of the reaction, regained, the group in question is assumed to play a positive role in the biological activity of the said protein. Of the several types of groups located in the side-groupings of the protein molecules SH,  $\text{NH}_2$  and phenolic groups have received most consideration as possible active "centers" of enzymes, toxins, hormones, viruses, and antigens. The subject has been variously reviewed during recent years (Herriott, 1947; Olcott and Fraenkel-Conrat, 1947; Anson, 1945; French and Edsall, 1945; Landsteiner, 1945), and we can only briefly refer to the pertinent phases of the subject. It should at the start be pointed out that there is as yet no clear demonstration which of a given number of specific groups are essentially related to the biological activity of a protein. The data so far available would seem to indicate that a certain number of a given group situated near or at a certain configurational position, more than others, may play a positive role in regulating the activity of a given species of proteins.

**b. Reaction of Formaldehyde with Proteins and Amino Acids.** It is a classical immunological fact that the action of formaldehyde converts toxins into toxoids, products which are deprived of toxicity without loss of antigenic potency and capacity to combine with antitoxins. French and Edsall (1945) subjected a large number of studies on the reactions of formaldehyde with amino acids and proteins to a comprehensive treatment. It shows that formaldehyde frequently enters into an addition reaction with a compound containing an active hydrogen atom with the formation of a mono-hydroxy-methyl compound,  $\text{R-CH}_2\text{OH}$ , which can enter into a condensation reaction forming a methylene bridge,  $\text{R-CH}_2\text{-R'}$ . Thus, numerous groups found in amino acids, peptides and proteins are capable of undergoing addition and condensation reactions with formaldehyde.



With the *amino* group it forms mono- and dihydroxymethyl derivatives,  $R-NHCH_2OH$ ,  $R-NH(CH_2OH)_2$ ; with the *amide* group, hydroxymethyl,  $R-CO.NHCH_2OH$ , and, at elevated temperatures, methylene diamide,  $CH_2(R-CONH)_2$ ; with one *imino* group, hydroxymethyl,  $R_2N-CH_2OH$ , and with two *imino* groups, methylene compound,  $(R_2N)_2CH_2$ ; with the peptide linkage, hydroxymethyl groups,  $-CO.N(CH_2OH)-$ ; with the *guanidino* group, a drastically changed arginine derivative; with the *hydroxy (alcoholic)* group, acetals,  $R-O-CH_2OH$ , and hemi-acetals,  $(R-COO)_2CH_2$ ; with the *sulfhydryl* (SH) group, thio analogs of acetals,  $R-S-CH_2OH$ , and hemiacetals,  $(R-S)_2CH_2$ . Action of formaldehyde on tryptophane, tyrosine, phenylalanine, and histidine leads to the formation of additional rings.

Fraenkel-Conrat and Olcott (1948) reported that under conditions of pH and temperature which are used for tanning and for the preparation of toxoids and vaccines, formaldehyde introduces a methylene bridge  $-CH_2-$  between amines on the one hand and the reactive groups of phenolic and imidazole rings on the other. The linkage is resistant to acid hydrolysis. Condensations joining indoles, amines, and formaldehyde under similar conditions may occur with the  $-NH$  group of the indole ring.

Very careful analytical determinations (Nitschmann and Hadorn, 1943) have shown that formaldehyde can partially (ca. 35 per cent or more) be removed from formalized casein by prolonged washing at room temperature. In combination with a protein, formaldehyde, therefore, exists in a form which is less firmly bound, or readily removable, and in another form which cannot be readily removed. The point was made that it is impossible, at present, to differentiate the chemical groups involved in these two forms of binding.

Velluz (1938) reported that in tetanus toxin formaldehyde combines with tryptophane forming an irreversible heterocyclic three ring compound, transforming the toxin into a new antigen. Pappenheimer (1938) reported that diphtheria toxin treated with low concentrations of formalin in alkaline solution forms an irreversible combination. The number of acetylated free amino groups corresponded to the number of  $\epsilon$ -amino groups of lysine present (5.3 per cent). In converting the toxin into toxoid about 40 per cent of the total free amino nitrogen was found still free. Eaton (1937) reported that 30 per cent of the amino



nitrogen of the toxin is slowly and irreversibly bound. In view of the irreversibility of the union of the toxin with formaldehyde, Pappenheimer considered this reaction not to be due to the mere formation of methylene linkages to the nitrogen. Hydroxymethyl compounds resulting from this reaction with free amino groups are unstable combinations (French and Edsall, 1945) and should therefore be reversible.

While with low concentrations of formaldehyde the toxin did not lose the ability to flocculate with antitoxin, the higher concentrations caused the destruction of antigenic properties. Horsfall (1934), and Jacobs and Sommers (1939) also reported serologically demonstrable changes with formalized proteins involving combinations other than with free amino groups.

The reviewers of the reactions with formaldehyde point out that the types of reactions it enters into is governed by the  $H^+$  and  $OH^-$  concentration, period of treatment and the type of protein treated. In neutral solution, the immediate reaction with proteins is a reversible combination with the free amino groups. With longer time of reaction, as in the case of the preparation of toxoids and vaccines, the formaldehyde slowly becomes more firmly bound with decrease of amino nitrogen; under these conditions, not only the amino, but also the indole, amide, and guanidyl groups react with formaldehyde, forming cross-linkages and more stable combinations and, therefore, antigenically modified proteins. There has been indication that formaldehyde reacts also with sulfhydryl groups (Anson, 1945).

In view of the many complications resulting from the action of formaldehyde on the various groups in the protein molecule, it is impossible to correlate any specific group in the protein molecule with its loss of viral, enzyme, toxic, serological and antigenic properties.

c. **Acylation of Proteins.** The principal reagents used for the acylation of proteins are ketene [ $(H_2C-C-A)$ ] and acetic anhydride  $(CH_3CO)_2O$ . Ketene has been extensively used with aqueous protein solutions. At a pH above 5.0, ketene reacts with  $NH_2$ , SH and tyrosine-OH groups in proteins. With  $NH_2$  groups it yields protein-NH-COCH<sub>3</sub>.

Acylation at pH 5.5 of the amino groups of pepsin causes no inactivation (Herriott, 1935). The number of amino plus tyrosine groups covered was less than the number of acetyl groups, indicating that some other protein groups had reacted. It has been found that ketene reacts

also with tryptophane (Herriott, 1935), and -SH groups (Fraenkel-Conrat, 1944). With native egg albumin, the reaction is faster with the  $\text{NH}_2$  than the SH group. In lactogenic hormone (Li and Kalman, 1946) the phenolic groups react with ketene faster than do free amino groups; in insulin amino groups are attacked more rapidly than phenol groups (Stern and White, 1938); and in parathyroid hormone (Wood and Ross, 1942) both the amino and phenolic groups are only about 40 per cent acetylated. In diphtheria toxin (Pappenheimer, 1938) the presence of amino groups of different reactivity with ketene has been indicated. Extensive treatment of tobacco mosaic virus with ketene fails to acetylate all the amino groups (Miller and Stanley, 1941).

Boor and Miller (1939) found that freshly ketenized gonococcus still retained enough toxin to kill two out of six mice, but after standing a week in the cold, the preparation killed six out of six mice, indicating the reversibility of the reaction of ketene with gonococcus. After short acylation at pH 6 to 7 with ketene (Goldie, 1937; Pappenheimer, 1938) diphtheria toxin lost its toxicity without losing its ability to combine and flocculate with antitoxin. During the detoxication a number of free amino groups were acetylated, corresponding closely to the number of  $\epsilon$ -amino groups of lysine present (5.3 per cent). Acylation also of the tyrosine-OH groups caused the loss of the ability of the toxin to combine with antitoxin. Little and Caldwell (1942, 1943) found that acetylation of the amino groups of  $\alpha$ -amylase with ketene deprived it of enzyme activity. Sulfhydryl and phenolic groups (tyrosine) groups were of little, if any, significance. Amylase was also inactivated by formaldehyde, phenylisocyanate and nitrous acid, which indicated that the primary amino groups were involved. On the other hand, Sizer (1945) finds that the action of ketene, nitrous acid, phenylisocyanate, formaldehyde, oxidants and reductants on chymotrypsin causes no inactivation, indicating that the primary amino, sulfhydryl or disulfide groups are not required for chymotrypsin activity. In tobacco mosaic virus, ketene or phenylisocyanate have been reported (Schramm and Müller, 1940, 1942) to react first with the free amino groups; later the phenol and indole groups are affected. Only the reaction with phenol and indole groups has been observed to be associated with loss of infectivity. The disappearance of the  $\text{NH}_2$  group with these reagents is said to be without any effect on the infectivity of the virus. Alkaline treatment is expected to hydrolyze the

acetylated phenolic groups. Failure of this treatment to reactivate the acetylated virus, however, led them to conclude that the inactivation of the virus was not due to acetylation of the phenolic groups. Miller and Stanley (1941) reported that about 70 per cent of the amino groups and 20 per cent of the phenol groups of tobacco mosaic virus could be acetylated without loss of activity.

Discussing the results of various studies, Olcott and Fraenkel-Conrat (1947) point out the possibility that also some of the aliphatic hydroxyl groups, and groups other than those concerned above, might be involved in the treatment of proteins by ketene. It would thus appear that ketene falls short of being a good protein reagent: its action is not specific; it falls short of acetylating completely any or all of the reactive groups; it involves difficult analytical manipulation; it tends to surface denature sensitive proteins; it appears to be extremely toxic, and the racemization of asymmetric carbon atoms of proteins has been indicated. Under these circumstances, the effects resulting from the ketenization of proteins leaves unexplained the specific relationship of various groups with the biological specificities of proteins.

**d. Iodination of Proteins.** Iodine has often been used as an oxidizing agent in the study of HS-enzymes. In dilute acid solutions, high iodine concentrations specifically oxidize SH-groups. In neutral and alkaline solutions, iodine substitution in the tyrosine groups of proteins occurs. In either treatment, both oxidation and substitution can take place. Upon iodination of proteins in strong ammoniacal solutions, under conditions which have been employed in studying the antigenic properties of proteins, additional impairment such as loss of species specific properties of the whole molecule would be expected to occur. It has been found that the rate of iodination is associated with the degree of denaturation. The rate of iodination of native protein is slower. In urea solution, which favors denaturation, iodination is more rapid, indicating an increased availability of the tyrosine groups for iodination with increasing denaturation. Iodination of imidazole groups (histidine) of proteins, and indole groups (tryptophane) may occur in excess of iodine and prolonged treatment.

Iodination of horse serum globulin in alkaline medium, when presumably all tyrosine is substituted in the 3 and 5 positions, caused loss simultaneously both of the ability to react with, and the ability to produce anti-species antibodies (Klecowski, 1940b). On the other

hand, the treatment of proteins with phenylisocyanate, forming  $R-NH-CO-NH-\langle \text{ } \rangle$  derivatives, or with formaldehyde only slightly reduced their affinity toward homologous antibodies. This was interpreted to indicate that amino groups of the antigenic proteins are not involved in combination with antibody (Kleczowski, 1940). As may be recalled from the preceding discussion on acetylation of proteins, the acetylation of the OH groups of tyrosine in diphtheria toxin was reported to cause the loss of the toxin's ability to combine with antitoxin (Pappenheimer, 1938). Due to technical difficulties, the loss or presence of the original species specificity of the acetylated toxin could not be determined. In connection with the results with iodinated proteins of Kleczowski, it must be remembered that substitution in positions 3 and 5 of tyrosine in proteins with diazonium haptenic radicals does not cause loss of the ability of the substituted proteins to produce anti-species antibodies.

Anson and Stanley (1941) reported that the treatment of tobacco mosaic virus with iodine, causing abolition of the sulfhydryl groups does not inactivate the viral activity. If enough iodine is added to the virus, or if the reaction is carried out at a sufficiently high temperature, converting the tyrosine groups into di-iodotyrosine groups, the viral activity is lost. Sizer (1945) found that likewise the activities of chymotrypsin and phosphatase are destroyed if tyrosine groups are destroyed. Strong oxidants, likewise iodine, inactivated chymotrypsin, involving the oxidation of its tyrosine groups. Herriott (1937) reported that iodinated pepsin is practically inactive when the number of iodine atoms per molecule of pepsin is 35 to 40. Since there are 16 tyrosine residues (mols) per mole of pepsin, the number of iodine atoms found in iodinated pepsin corresponds to complete di-substitution of all the tyrosine molecules, or three to eight atoms of iodine more than required by theory.

In evaluating the above considered data one must, no doubt, keep in mind that in addition to the substitution of iodine in the tyrosine molecules, other substitutions and the oxidation of SH groups, under the experimental conditions used, and also the denaturation of the protein molecule, would be expected to occur.

**e. Reactions with Sulfhydryl and Disulfide Groups of Proteins.** It is known that the  $-SH \rightleftharpoons -S-S-$  relationship in certain enzyme



proteins plays a role. This relationship to enzyme activity has been discussed by Barron (1943), and to the denaturation and properties of proteins, by Anson (1945). The role of the SH group in the activities of succinoxidase, phosphoglyceraldehyde dehydrogenase, phosphoglucomutase, and the pyruvate oxidase system has been emphasized. It is common practice to activate papain by certain reducing agents, suggesting, perhaps, a role for SH groups in this enzyme.  $\beta$ -Amylase (from barley and malted barley) activity was found by Weill and Caldwell (1945) to be associated with SH groups. The inactivation of the enzyme by dilute iodine, ferricyanide and cupric ion was reversed by hydrogen sulfide. The enzyme was irreversibly inactivated by treatment with iodoacetamide, which, as is known, forms an irreversible derivative with the SH groups.

Micheel and Bischoff (1937) reported that the reduction of crotoxin by means of cuprous oxide, cysteine and a stream of oxygen inactivates the venom by converting the R-S-S-R to RSH groupings. Sulfurous acid alone was capable of inactivating the venom in this manner. A crystalline product derived from crotoxin (rattlesnake venom) was found to contain 4 per cent sulfur existing in -S-S- linkages. (Slotta and Fraenkel-Conrat, 1938). Reduction with cysteine of this product likewise destroyed reversibly the toxic activity in a manner comparable to the reversible inactivation of insulin (du Vigneaud *et al.*, 1931-1932). De (1940) observed that cobra hemolysin is reversibly inactivated by cuprous oxide or organo-mercurials and reactivated by hydrogen sulfide and reduced glutathione. Benzoquinone depressed the activity of purified hemolysin in 10 minutes; this was partially regenerated by hydrogen sulfide. Henry (1939) had found that reduced glutathione given either *in vitro* or *in vivo* counteracted the anticoagulant activity of cobra venom. Tetsch and Wolff (1937) analyzed various animal and insect toxins and found the following correlation between the sulfur content and toxicity.

TABLE VII

Species	% Sulfur	Fatal $\gamma$ poison/g. mouse
Bee poison	2.6	10.0
Scorpion poison	3.8	0.7
Cobra venom	5.5	0.15 to 0.12
Crotoxin (rattlesnake)	3.6	0.7



Velluz (1938) observed that carbon disulfide detoxifies tetanus toxin, not diphtheria toxin, the difference being ascribed to the absence of SH groups in the latter. Diphtheria toxin contains 0.75 per cent sulfur, but fails to give the nitroprusside test for SH (Pappenheimer, 1937). This amount of sulfur corresponds to about 20 molecules of cysteine per molecule of toxin of 72,000 molecular weight.

Pillemer, *et al.* (1938) found that urease oxidized by cuprous oxide and air is reactivable with reducing agents,  $H_2S$  and KCN. Although oxidized urease has lost its specific reactivity with antiserum to crystalline active urease, oxidized and reduced urease elicited similar antibodies. This was explained by the fact that whole blood was unable, but tissue extracts were capable of reactivating oxidized urease, which indicated that the oxidized urease is made specifically antigenic in the animal tissue. Pillemer, *et al.* (1939) converted keratins (wool, chicken feathers, human hair) into their sulfhydryl derivatives (kerateines), and the latter were oxidized to obtain di-thiol derivatives (meta-keratin). Immunologically it was found that species specificity is an individual characteristic of the keratin. Optimal species specificity was demonstrated only when the reduced keratin (kerateine) was allowed to react with the antiserum prepared by the injection of the homologous kerateine. Thus, immunological differences among the keratins were detectable depending on the state of oxidation or reduction of the SH groups in the proteins. Using a similar approach, Ecker and Pillemer (1940) found immunological species differences between the ocular lens proteins of chicken and fish (pike). Similar proteins from swine and sheep lenses behaved as if identical. Reduced swine lens protein contained 8.5 per cent sulfur, and those from the other three animals from 4.3 to 5.2 per cent. In anaphylactic tests, Markin and Kyes (1939) found species differences between the lens proteins of dog and beef and that from the pigeon lens.

The above results would seem to show that sulfhydryl and disulfide groupings play significant rôles in certain enzyme reactions as well as in the specificity of certain antigenic proteins and toxins.

## 2. Identity of the Nature of the Inhibition of Enzymes by Homologous Antibodies with Antigen-Antibody Reactions

A consideration of the results of various studies on the specific side groupings of proteins in relation to their various biological activities fails to establish a direct causal relationship. It is true that modifications in certain groups result in reversible inactivations, but what other changes underlie such modifications are not known. It must be remembered that the integrity of the whole protein molecules to which various side groups are attached, and not the groups by themselves, determines the complex mechanism of the biological activities of proteins. For example, the relation of the SH groups in succinoxidase does not bear even a suggestion of similarity to their rôle in the activation of papain or the activity of pneumococcal hemolysin. We do not know as yet what particular configuration or groupings in one protein enable the heme group to function as cytochrome oxidase, and in another protein enables it to act as a catalase, or cytochrome c. What makes a certain protein to enable pyridine-adenine-dinucleotide to function as coenzyme for lactic acid dehydrogenase and another protein enables it to act as coenzyme for phosphoglyceraldehyde dehydrogenase?

Similar difficulties arise when we consider the groupings involved in the combinations between antigens and antibody. There is no doubt that certain specific groupings in these reactants make them mutually attractive and a union takes place. The suggestion of Heidelberger and Kendall (1929), and the experimental data provided by the studies of Chow and Goebel (1935), and Goebel and Hotchkiss (1937), as we have discussed elsewhere, contribute to the support of the idea that the positively charged  $-\text{NH}_3$  groups in antibodies and the negatively charged  $-\text{COOH}$  groups in antigens might be involved in the combinations between antigens and antibodies. According to Pauling (1945) hydrogen bond linkages may arise from reactions involving positively charged imino,  $=\text{NH}$ , and negatively charged carboxyl,  $-\text{COO}^-$ , groups forming  $\text{R}-\text{N}-\text{H} \cdots \text{O}-\text{CO}\cdot\text{R}$  bonding. It is suggested that the forces of these bondings play a rôle in keeping the proteins in their specific configurations. From the results of haptenic inhibitions of antigen and antibody reactions, it has been suggested (Pressman, Bryden and Pauling, 1948) that the principal forces of

attraction between a positive charge in the antibody and negative charge of the carboxyl of a haptene, presumably forming a hydrogen bond, are responsible for their union. In his discussion on the denaturation and properties of various protein groups, Anson (1945) considers the hydrogen bond theory plausible on general chemical grounds, but it has not as yet any direct experimental basis.

It is evident also in these reactions between the positively charged ammonium groups of antibodies and negatively charged carboxyl groups of antigens, with or without the formation of hydrogen bonds, that these side groupings do not themselves carry the specificity of the reactions of the complex reactants to which these side groupings are attached. There is no specific information as to what properties of the configuration of an antigen enable its carboxyl groups to react specifically with the ammonium or imino groups of the homologous antibody, and do not permit them to react with the same groups in heterologous antibodies.

The question of whether an antibody which specifically inhibits the biological activity of an antigen contains specific configuration evolved in response to the stimulus by the active groups of the specific protein molecule, or whether the observed inhibition is the result of a secondary reaction associated with the union of antigen and antibody may now be considered. It is a known fact that the toxicities of antigens can be eliminated with non-specific agents without loss of their ability to produce antibodies possessing the ability of combining with both the inactive and active forms of the antigen. The unanswered question is the mechanism by which inactivated antigen is capable of producing an antibody which neutralizes the toxicity or the enzyme activity of the antigen. Ramon (1943) inquiring into this question experimented with papain. As Achalme (1901) had previously reported, Ramon observed that papain acts like certain bacterial toxins and venoms. The papain solutions filtered through porcelain and injected subcutaneously into guinea pigs, rabbits, or a horse produced local disorders. Oedema and inflammation were followed by the formation of a scar. Papain in large amounts caused the death of animals. Treated with formaldehyde at 45°C. papain lost its *in vitro* enzyme activity and *in vivo* toxicity. Detoxified papain produced antibody which flocculated and neutralized the toxicity and the known activity of the enzyme, in a manner comparable to the properties of diphtheria

toxin, toxoid and antitoxin. He was unable to offer any explanation for this anomalous reaction. Stanley (1936) made similar observations with tobacco mosaic virus and arrived at the conclusion that the precipitin reaction which has been used as a measure of virus activity, may not be used unreservedly as a measure of virus activity, for in the case of inactive protein there is no correlation between the precipitin titer and virus activity. His inactivated virus produced immune sera which neutralized the viral activity.

In the consideration of any of the systems belonging to the above category, we must look into the presence or absence of a direct relationship between enzyme and antibody producing activities of the proteins and their abilities to combine with respective antibodies with or without the neutralization of their biological activities. While combination of an antigen with its homologous antibody and other combinations with serologically non-specific inhibitors are stoichiometrical reactions, the mechanism which governs the enzyme and antibody producing activities of proteins are of catalytic nature. Stable stoichiometrical combinations with the specific active groups would be expected to block simultaneously all of the above named activities of a given protein. It is, perhaps, for this reason also that in the form of antigen-antibody complex an antigen may fail to produce a satisfactory, if any, amount of antibody. In other words, when the specific groups are completely blocked by specific antibody not only enzyme activity, but also its ability to catalyze the production of specific antibody is inhibited. After reviewing the literature and on the basis of his own experimental findings, Olitzki (1935) reported that when the receptor groups of an antigen are saturated with antibody the treated antigen is deprived of its capacity to develop specific antibodies. He found that the injection of sensitized antigen together with free antibody suppresses the formation of antibodies to 10 to 20 per cent of the amount obtained with injections of antigens without serum and the rate of reproduction is much slower.

When larger amounts of antibodies are added, then the formation of agglutinins can be completely stopped. As sensitized bacteria *in vivo* and *in vitro* can be phagocytized, sensitized pneumococci and toxin-antitoxin complexes can be attacked by proteolytic enzymes liberating the cells or toxins without loss of activity, the failure of antigen in the form of the antigen-antibody complex to stimulate the production of



antibody would not appear to be due to an *in vivo* blocking of the antigenic groups from exercising their activity. There seems, therefore, to exist a striking interrelationship between the specific combining abilities of antigens and their catalytic activities. There is, however, the fact that when an antigen is proteolytically reduced to split products the enzymic and antigenic properties are lost without parallel loss of combining abilities. The split products merely function as haptens. Here again, the point to be remembered is that the catalytic activity of the intact molecule is the principal factor which enables its potential haptenic groups to invoke complementary parts in the antibody molecule during its synthesis.

The interrelationships cited do not appear to account for the loss of enzyme (or toxic) activity without loss of the ability to produce neutralizing antibody when the toxin or enzyme is treated with non-specific agents such as ketene or formaldehyde. This discrepancy would seem to be more apparent than real. The following possibilities can be considered to account for this discrepancy:

*First:* The neutralization of the enzyme or toxic activity of a protein by its specific antibody may be a secondary effect related to the union of the protein as antigen with the antibody which has been produced in response to the inactivated but antigenic protein. If formaldehyde, ketene etc., can cause inactivations, one could postulate that inactivation of the enzyme and toxic properties would automatically result when the antigen-antibody combining reaction occurs. Such combinations produce neutralization of the respective negative and positive charges, and a decrease in the energy content of the reactive groups. Consequently, possible deleterious effects on other regions of the active protein molecules might transform the enzyme molecule into an inactive form in the combined state.\* There are no experimental data to support or to refute such an interpretation of the observed effect. The discussed properties are so interrelated that it is difficult to characterize which is cause, which is effect. As in the oxidation of SH groups, or the reduction of  $-S-S-$  groups resulting in the

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\*Stanley (1936) reported that the precipitation reaction, which has been used as a measure of virus activity, may not be used unreservedly for this purpose.

Kassanis (1943) reported that normal and heterologous sera cause marked neutralization of plant viruses—tobacco mosaic virus, tomato bushy stunt virus and two cultures of tobacco necrosis viruses. The additional specific effect of homologous antisera was small in comparison. Unless sera were kept frozen their non-specific neu-



inactivation of the enzymes or toxins, one cannot define what configurational and other more critical changes in the whole molecule precede or follow such reactions. We must, therefore, inquire into other possibilities which may account for the above mentioned discrepancy.

*Second:* The second possibility is the reactivation *in vivo* of the *in vitro* inactivated enzymes and toxins, etc. The amount of the reactivated molecules produced at a given time might be insufficient to produce noticeable toxic effects but sufficient to produce specific antibodies. The data concerning this point are not as yet adequate, but whatever can be cited is strongly suggestive of this process. For example, urease oxidized with dilute iodine is inactive. This inactivation, probably involving the oxidation of SH groups, is reversed by sulfhydryl groups.

As discussed above, Pillemer, *et al.* (1938) showed that although oxidized urease has lost its specificity to react with antiserum to crystalline active urease, both the oxidized and the reduced urease produced a similar antibody. The ability of the oxidized urease to produce such a specific antibody was attributed to the fact that tissue extracts were capable of reactivating oxidized urease, showing that the oxidized urease regains its lost specificity in the animal system. Similarly, the detoxification of snake venom involves the reduction of di-thiol groups into sulfhydryl groups. *In vivo* the reduced molecule can regain to some degree the original form. The reaction of formaldehyde with the imino, amino, amide, hydroxyl and sulfhydryl groups are reversible reactions, though some are more stable than others. Which of these reactions predominate in the conversion of toxin into toxoid is not known. It is assumed, but contested, that formaldehyde reacts principally with amino groups. If this is the predominant reaction the resulting hydroxymethyl compounds are unstable and reversible. The toxoid molecules can be considered subject to reversion *in vivo* to the original molecular form. Acetylation with ketene likewise would seem

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tralizing power fell rapidly on stirring. All heterologous antisera reduced infectivity more than normal sera stored similarly.

Precipitating antibodies did not appear to be responsible for neutralization. No correlation was found between precipitation titre and neutralization power, and removal of precipitins did not affect neutralization power. Only quantitative differences were found in behavior between homologous and other sera; the infectivity of all virus serum mixtures was regained by dilution.

to produce unstable derivatives. As referred to above, Boor and Miller (1939) found that kетенized gonococci after standing a week in the cold fully regained their toxicity to mice.

In the light of what we know about the above discussed reversible inactivations, one is inclined to justify the assumption that this process occurs provided the protein under consideration has not been subjected to greater changes.

The many observed inhibitions of enzymes and toxins by their specific antibodies may lend themselves to two types of interpretations. These inhibitions may be due to direct combination between the specific groupings in the enzyme and anti-enzyme antibody molecules. If such is the case it is immaterial whether or not the substrate molecule can penetrate the immune complex to reach the site of the active enzyme groups. The substrate could not be activated under these conditions. Or, the antibody molecule may not contain groups specific for the enzyme, but the antibody molecules occupying positions on the surface of the enzyme may be so closely packed that the substrate molecules are incapable of reaching the site of the active enzyme or toxin groups.

There are no experimental data to show that antibody molecules on the surface of antigen constitute a mechanical barrier to the substrate molecules. Before such an argument can be deemed worthy of consideration it would be desirable that experimental results be provided. One may perhaps be able to work out conditions for the treatment of the enzyme-antienzyme complexes with acetylating and other agents containing tagged isotopic atoms. On separating the antigen from the antibody, the relative amounts of the tagged agents in each component could be estimated. Using the size of the reagents as a measure for the space available between the antibody molecules on the surface of antigen one may be able to gain some information concerning this question.

On the basis of various experimental data it would seem difficult to conceive that antibodies combining with an enzyme molecule constitute a wall impermeable to the specific substrates. In the reaction of diphtheria toxin with antitoxin, in the presence of extreme excess of the latter, at least eight molecules have been shown to combine with one molecule of toxin. The composition of toxin-antitoxin soluble complex in the zone of toxin excess has been reported to consist of only one

antitoxin molecule for two of toxin (Pappenheimer, 1940; Pappenheimer, Lundgren and Williams, 1940). Boyd (1947) has tabulated molecular compositions of various antigen-antibody complexes. In the presence of *extreme excess of antibody*, serum albumin can combine with six rabbit antibody molecules; one molecule of thyroglobulin (mol. wt. 650,000) combines with forty rabbit antibody molecules; ovalbumin with four (horse) or five rabbit antibody molecules; and nine hundred rabbit antibody molecules with one of tobacco mosaic virus. The high molecular composition of certain antibodies with the respective antigens is understandable if we take into consideration the molecular weights of these antigens. The molecular weight of tobacco mosaic virus is 60 million. The molecular weights of diphtheria toxin and serum albumin are about 70,000, and that of ovalbumin about 40,000.

Since the size of rabbit antibody globulin to various antigens is constant, the critical factor in the molecular composition of antigen and antibody complexes is the molecular size and the surface areas of antigens. The ratios of the molecular weight of the tobacco mosaic virus to that of the serum albumin or diphtheria toxin is about 860. One virus particle combining with 900 rabbit antibody molecules would be equivalent to a combination of one molecule of antibody and one virus unit of 70,000 molecular weight. This approximation would be valid if the shape of the virus and that of the serum albumin or diphtheria toxin are of comparable dimensions. The ratio of the major to minor axis of the diphtheria toxin is 4.7, and that of the virus is 18. Weight for weight, virus particles may therefore possess a three times greater surface area. Using this rough relationship, one can see that only a very few antibody molecules can combine with one sub-molecular unit of virus antigen. Under these conditions, the formation around the antigen molecule of a barrier impermeable to a substrate does not appear to be probable.

Let us examine other data which would seem to throw some light on this question. It had been assumed that when antibodies combine with microorganisms, causing clumping or agglutination, they simply reduce the effective surface relationship between the enzyme and substrate (Taliaferro, 1948). Under these conditions it had been assumed that substrates are prevented from reaching the enzyme sites. Sevag and Miller (1948) found that agglutinated pneumococci with or with-

out complement use just as much oxygen as non-agglutinated or control systems. The results with *E. typhosa*, 0-901 strain, were similar to those obtained with pneumococci. These findings show that the layer of antibody specifically deposited on the surface or cell-wall of microorganisms, with or without agglutination, do not constitute a mechanical or physical barrier to the penetration of glucose or glycerin to the active sites of enzymes if the latter are not inactivated by an antigen-antibody combination. In a study with *Salmonella* Harris (1948) obtained results similar to that discussed above. The question of whether or not protein or starch molecules can squeeze themselves to the sites of the specific enzymes of agglutinated bacteria through the layer of deposited antibody molecules remains to be asked.

In this connection an observation by Feiner, *et al.* (1946) is of considerable interest. They studied the ability of lysozyme to attack a substrate (antigen) precipitated by homologous antibody. They observed an unmistakable difference in the appearance between the untreated and enzyme-treated immune precipitates. The control, untreated precipitates remained as opaque white pellets, whereas in the treated series they appeared as translucent vacuolated material closely adherent to the bottom of the tube and markedly diminished in size. Antibody was released as a result of lysozyme action. They concluded that lysozyme is capable of attacking the organism, or its mucopolysaccharide, as substrate when either is combined with antibody. Lysozyme has a molecular weight of 15,000 to 18,000 and is a protein. Its substrate, mucopolysaccharide, is antigenic and combines with antibacterial serum. The ability of lysozyme to depolymerize this antigen (substrate) when in combination with antibody molecules shows that the latter occupying positions on the antigen molecule leave free spaces through which lysozyme can readily make contact with the enzyme-susceptible groupings, or that mutual affinities between an enzyme and substrate cause displacement of the antibody molecules from their combining sites on the antigen. Whichever explanation is considered more plausible, the fact remains that either the antibody molecules are incapable of forming a wall around or on the surface of an antigen which is impermeable to a protein of 15,000 to 18,000 molecular weight, or that the enzyme competes with the antibody for the same site on the substrate (antigen) molecule and is capable of displacing it. If we consider these relationships even in a most conservative man-



ner, the immune inhibitions of the metabolism of the small molecular weight substances cited below do not appear to be due to mechanical obstructions resulting from or associated with antigen-antibody reactions.

In other sections of the present treatise, the inhibition by homologous antibodies of various enzymes which specifically catalyze the metabolism of pyruvate, penicillin, tyrosine, luciferin, lecithin, urea, sucrose, amygdalin, tributyrin lipids, and d-glyceraldehyde-3-phosphate will be described. These substances are small molecular weight substances. Kirk and Sumner (1931) demonstrated that rabbits immunized against urease could survive 1000 lethal doses of urease. This result shows that in the animal the system urease-anti-urease complex is in an enzymatically inactive state, or that the wall set up by the anti-urease molecules around the urease molecule is so tightly packed that even an urea molecule of 60 molecular weight is incapable of reaching the active site of the enzyme. As discussed above, a few molecules of an antibody combined with an antigen molecule do not appear to us to be capable of forming a solid wall impermeable to urea molecules.

Lipmann and his associates (Zamecnik, Brewster and Lipmann, 1947) have demonstrated that lecithinase of *Cl. welchii* is completely inhibited by homologous antibody. The reactions between the lecithinase and antibody, and that of the enzyme and its substrate lecithin are competitive. When the substrate is first added to the enzyme antibody fails to inhibit the enzyme action. Conversely, when the antibody is added to the enzyme first, the enzyme fails to catalyze the substrate added last. Lecithin is known to combine with an antigen-antibody complex (Horsfall and Goodner, 1935, 1936). The inability of lecithinase, when in combination with antibody, to hydrolyze lecithin is probably not, therefore, due to an absence of combining affinity of lecithin for the complex. Is this failure of the enzyme due to the failure of the reactive groups of the enzyme and substrate to meet each other? Is antibody incapable of having access to the combining groups of the antigen molecule when the latter is reacting with its specific substrate? Since the enzyme-substrate combination is a continuously reversible one, and since the antigen-antibody combination is also very rapid and produces a relatively stronger union, or only a very slightly reversible one, one is inclined to assume that there should be repeated occasions



when the enzyme is free for combination with the specific antibody. Such combinations being comparatively non-dissociable, the concentration of the enzyme would be markedly reduced, in other words, one should notice measurable decrease in the degree of enzyme activity. Since, in experiments where the enzyme and the substrate are first brought together, such decreases in activity are not observed, it would seem that the affinity between the enzyme and substrate is far greater than that of the enzyme and the antibody. A somewhat different picture is gained from the results of Housewright and Henry (1947) obtained from a study of penicillinase-penicillin-antipenicillinase interactions (see p. 332). In this, as in the lecithinase studies, the substrate penicillin was incapable of displacing the specific inhibitor, antipenicillinase, from its combination with penicillinase. On the other hand, penicillinase which had been in contact with penicillin showed affinity for antipenicillinase which was twice as great as that shown for penicillin.

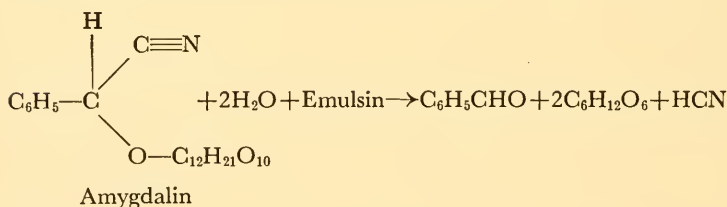
### C. ANTIBODY AGAINST CARBOHYDRASES

A review of the studies on immune reactions reveals that in the earlier period of the development of the science of immunology investigators held the point of view that enzymes played an important rôle in the pathogenesis of infectious diseases. They may have thought in the inner recesses of their minds that immunity against infectious diseases might in some way be related to anti-enzyme immunity. It is perhaps for this reason that among earlier immunological studies there are numerous reports on the subject of anti-enzyme immunity. While methods for the study of toxin-antitoxin, venom-antivenom reactions, and those pertaining to the study of various phases of anti-bacterial and anti-hemolytic immunity have been developed and perfected, the development of reliable methods of preparing enzymes for similar studies has been progressing at a slower rate. The more subtle nature of the enzymes and the ease with which their activity is reduced or abolished may have contributed in part to this slow progress. Despite these shortcomings, a beginning was made as early as 1893 when Hildebrandt reported the first study of its kind on the formation of antibody against emulsin. We will therefore begin with the description of the study of this early investigator.

## 1. Antibody Against Emulsin

In 1893 Hildebrandt reported that the injection of emulsin prepared from almonds into rabbits caused loss of weight and finally death. Injecting 0.3 to 0.9 g. of emulsin rectally into rabbits daily, and increasing the amount of emulsin to 1 g. during the second week the animal did not lose weight and no other pathological symptoms were observed. Injecting 1 g. of emulsin rectally in one dose caused, however, the death of control rabbits within 24 to 48 hours. On the basis of these results, he believed the animal which received emulsin in small doses during one week had developed local immunity. In subsequent experiments he resorted to the immunization of rabbits by subcutaneous injections. He first injected an initial dose of 0.01 g. of emulsin and increased the dose gradually to 1 g. (dissolved in 20 ml. of 0.6 per cent sodium chloride solution). No injury was suffered by the rabbit. In contrast, the control rabbits succumbed within one week after receiving 0.1 g. and two days after receiving 0.5 g. of emulsin.

The action of emulsin on amygdalin produces hydrocyanic acid. The formation of this poison *in vivo* by injecting the enzyme and the substrate into a rabbit was proven by the instantaneous death of the animal with the symptomatology of cyanide poisoning.



The simultaneous intravenous injection of 0.03 g. of emulsin and 0.5 g. of amygdalin into a normal rabbit caused the death of the animal within less than one minute from cyanide poisoning. In contrast in a rabbit immunized against emulsin and treated as above, the cyanide poisoning did not appear until after a period of six to seven minutes had elapsed. Hildebrandt expressed his observations as follows: "Diese Thatsache spricht zweifellos dafür, dass es sich bei der Emulsinfestigkeit nicht bloß um eine Immunität der Zellen des Organismus gegen die Giftwirkung des Fermentes handelt, sondern dass eine Art Gegen-

gift in den Saften des Körpers erzeugt sei, welches das Ferment unwirksam und daher wohl auch sonst unschädlich für das Thier macht."

The six to seven minutes' delay in the appearance of an otherwise instantaneous *in vivo* reaction fatal to the rabbit was regarded by Hildebrandt as sufficient evidence in support of the existence of anti-emulsin.

**a. Inhibition of the Amylase Activity of Emulsin Preparation by Anti-Emulsin Serum.** To prove further that he was dealing actually with immune inhibition of emulsin activity *in vivo*, Hildebrandt proceeded to determine the anti-amylase activity of the immune rabbit serum prepared against emulsin. The emulsin preparation hydrolyzed not only the glucosides—amygdalin, salicin, phlorizin, arbutin and coniferin—but also starch. In the presence of normal rabbit blood and emulsin the hydrolysis of starch produced 86.5 per cent reducing sugars within a period of twenty hours; in the presence of immune rabbit blood under similar conditions 39.5 per cent reducing sugars were produced. In separate experiments immune blood alone produced 38.5 per cent and emulsin alone 35 per cent reducing sugars. After making the necessary corrections for blanks he calculated that 34.5 per cent (47 per cent if compared with the normal blood) less reducing sugars had been produced in the presence of immune rabbit blood. Since the glucoside hydrolyzing activity of emulsin is due to  $\beta$ -glucosidase and this enzyme can now be prepared in relatively pure form, it might be of interest to undertake the production of antibody against  $\beta$ -glucosidase.

The production of immunity against the "emulsin" enzyme complex was also reported by Ohta (1913). Repeating the experiments of Hildebrandt he produced immunity in rabbits, the sera of which inhibited the hydrolysis of amygdalin by the emulsin preparation.

The hydrolysis of amygdalin was determined in a system containing 10 ml. of 2 per cent amygdalin solution, 0.1 ml. of 1 per cent emulsin extract, 1 to 4 ml. of immune or normal rabbit serum and 9.0 ml. of normal saline. The reaction mixture was then incubated at 37°C. for 24 hours under sterile conditions. The rate of the hydrolysis of amygdalin was determined by estimating the quantity of glucose produced after removing the proteins with colloidal iron. The results are summarized in Table VIII. The results show that anti-emulsin sera

inhibit the hydrolysis of amygdalin by emulsin. Since serum is a strong buffering medium the inhibitory effect due to a possible decrease in  $H^+$  concentration appears to have been eliminated in these comparative studies.

TABLE VIII

*The Inhibition of the Hydrolysis of Amygdalin by Anti-Emulsin Immune Rabbit Sera*

No. experiment	Reaction system	Glucose per cent	Inhibition per cent
1	Immune Serum I 1 ml.	51.5	27
3	Normal Serum I 1 ml.	52.8	
2	Immune Serum I 2 ml.	37.9	
4	Normal Serum I 2 ml.	51.8	
5	Control without serum	73.1	
6	Immune Serum I 4 ml.	36.6	56
7	Normal Serum I 4 ml.	82.5	
8	Immune Serum II 3 ml.	43.8	28
10	Normal Serum II 3 ml.	61.0	
9	Immune Serum II 4 ml.	43.5	
11	Normal Serum II 4 ml.	61.8	
12	Control without serum	69.5	
13	Immune Serum III 2 ml.	33.0	46
15	Normal Serum III 2 ml.	61.8	
14	Immune Serum III 4 ml.	32.2	43
16	Normal Serum III 4 ml.	56.4	
17	Immune Serum IV 2 ml.	25.2	47
19	Normal Serum IV 2 ml.	47.3	
18	Immune Serum IV 4 ml.	29.8	43
20	Normal Serum IV 4 ml.	52.3	

Bayliss (1912) immunized four rabbits intraperitoneally with emulsin in toluene water injecting 5 ml. of a 5 per cent emulsin solution at intervals of six days for seven or eight weeks. Eight days after the last injection the rabbits were bled and the sera were collected. The clear filtered solution of emulsin added to the sera produced a precipi-

tate. Normal sera did not form a precipitate with emulsin. If immune sera were added to either lactose, arbutin or amygdalin together with emulsin the hydrolysis was diminished or abolished. Despite these facts, Bayliss did not believe that the inhibition of the activity of the enzymes in emulsin was due to an immune reaction. In support of his belief, Bayliss also made the statement that: "Incidentally it is shown that emulsin is not of protein nature." The experimental data of Bayliss have been subjected to a critical analysis by us, particularly the results concerning the effect of  $H^+$  concentration on the reaction systems he used. We have found that Bayliss' results show a 79 per cent inhibition of the enzyme by immune rabbit serum which is due to emulsin-antiemulsin reaction.

Moreover, the objection of Bayliss that the variation of  $H^+$  concentration is responsible for the anti-enzyme inhibitory effect has been carefully investigated by various investigators (Lüers and Albrecht, 1926; Schubert, 1933; Smolens and Sevag, 1942, etc.); in all these and other studies, under carefully controlled  $H^+$  concentration the inhibitory effects of anti-enzyme sera are found directly related to antigen-antibody combination.

Dochez and Avery (1916) reported that antipneumococcal serum added to a culture of pneumococcus in inulin completely suspended the fermentation of the inulin. Heterologous immune serum delayed the reaction but did not entirely inhibit it. The fermentation of sugars and saccharose was not inhibited to the same degree as that of inulin. The rate of production of acid in cultures containing such easily fermentable sugars was markedly delayed by immune serum in the early hours of growth. The demonstration of the presence of anticarbohydrases in antipneumococcal sera capable of inhibiting the hydrolysis of inulin appears to corroborate the finding of Saiki (1907), who reported having produced antibody in animals against inulase. Dochez and Avery took blood serum from patients at intervals during an attack of lobar pneumonia and found that the serum exercised anti-enzymatic action similar to that of immune serum developed during the period of recovery from the disease. In view of the high degree of inhibitory action of the immune sera on the bacterial enzymes, they suggested that these properties play an important part in resistance and immunity to infection with pneumococcus.



## AMYLASES

## 2. Specificities of Amylases

Starches can be separated into at least two fractions, *amylose* ( $\alpha$ -amylose) and *amylopectin* ( $\beta$ -amylose). Amylose consists of unbranched chains of glucose residues combined in  $\alpha$ -1,4-glucosidic linkages. Amylopectin consists of both unbranched and branched chains of glucose units, and contains  $\alpha$ -1,6-glucosidic linkages as well, which are present at the point of branching.

Glycogen, unlike starches, is homogeneous in the sense that it consists only of branched chains. However, the  $\alpha$ -1,4-glucosidic linkage is the most important structural feature common to both starch and glycogen. The enzyme  $\beta$ -amylase acting upon glycogen attacks the outer branches composed of maltose units, yielding 47 per cent maltose and 53 per cent dextrin resistant to  $\beta$ -amylase. The outer branches of amylopectin are likewise degraded by  $\beta$ -amylase to the extent of 55 per cent to maltose, leaving residual dextrin resistant to this enzyme. Dextrin is composed chiefly of units of hexasaccharides. After the action of  $\beta$ -amylase on starch ceases, the resulting dextrin is attacked by  $\alpha$ -amylase. According to Myrback (1947),  $\beta$ -amylase attaches itself to free non-reducing end-groups of normally formed chains in which the glucose residues are united by the  $\alpha$ -1,4-glucosidic linkages. If, at a certain point in the molecule, the structure deviates from this pattern the enzyme action stops at this point. Since branching in amylopectin occurs on the *sixth* carbon of some of the glucose units in the chains (Meyer, 1943), these 1,6-linkages are probably responsible for stopping the hydrolysis at or near the points of branching. Thus,  $\beta$ -amylase splits the exterior unbranched maltose chains of starch, exposing branch points to the action of  $\alpha$ -amylase. The action of the latter enzyme makes branches of glucose units in  $\alpha$ -1,4-glucosidic union again available for the action of  $\beta$ -amylase until a junction (branch point) once more obstructs the decomposition.

According to Myrback (1947),  $\alpha$ -amylase is not only independent of free end-groups, but its action is successfully hindered by the proximity of end-groups. It has the capacity of attacking and rupturing any maltose linkage in a chain molecule of starch type, but the velocity is

greater for linkages at a distance from end-groups.  $\alpha$ -Amylase and malt-amylase free from maltase ( $\alpha$ -glucosidase) form glucose directly from dextrin, showing that glucose is formed not only from starch but also from short chain saccharides (4 to 6 glucose units) with maltose linkages ( $\alpha$ -dextrins).

Amylose, having solely unbranched chains of glucose residues, is hydrolyzed by  $\beta$ -amylase until the whole molecule is degraded to maltose units (for a general review of the subject see, Meyer, 1943; Hassid, 1945).

$\beta$ -Amylase has been obtained in crystalline form from sweet potatoes. This crystalline protein (17.48 per cent nitrogen) was free from  $\alpha$ -amylase activity (Balls, Thomson and Walden, 1946).  $\alpha$ -Amylase has been obtained from the pancreas likewise in crystalline form (Meyer, Fischer and Bernfeld, 1945). A molecular weight of less than 20,000 by the diffusion method has been calculated.

*$\alpha$ -Amylase.* Little and Caldwell (1942, 1943) sought to determine the nature of the active groups in pancreatic  $\alpha$ -amylase. Primary amino, sulfhydryl and phenolic tyrosine groups were the objects of their study as being possibly responsible for the activity of enzyme protein. Using ketene for acetylation, they established that the primary amino groups of the enzyme protein are essential to the activities of amylase. No evidence was found for the presence of free  $-SH$  groups in the active enzyme or for their importance to its activities. Amylase was not easily oxidized or reduced. Iodoacetic acid, which is specific for the sulfhydryl group of proteins, failed to affect the activity of the enzyme. Phenolic groups of tyrosine were of little, if any, importance to the activities of the amylase. Amylase was inactivated by formaldehyde, phenylisocyanate and nitrous acid, which are known to react with primary amino groups. Schwimmer and Balls (1949) have obtained  $\alpha$ -amylase of germinated barley (malt) in crystalline hexagonal prism. From osmotic pressure data a molecular weight of about 59,500 was calculated. One molecule of enzyme was found to hydrolyze 19,000 glycosidic bonds per minute. It appears that crystalline preparations require two calciums per molecule of  $\alpha$ -amylase.

*$\beta$ -Amylase.* Unlike  $\alpha$ -amylase, the activity of  $\beta$ -amylase from barley and malted barley was related to free  $-SH$  and phenolic tyrosine groups (Weill and Caldwell, 1945). Inactivation by nitrous acid was completely reversed in the early stages of the reaction at least, by sub-

sequent treatment with hydrogen sulfide. Only small loss of activity ensued on acetylation with ketene. That free sulfhydryl groups are associated with the activity of  $\beta$ -amylase are shown by the following tests: (a) positive nitroprusside test for free  $-SH$  group; (b) the inactivation by dilute iodine is reactivated by hydrogen sulfide; (c) significant reactivation by hydrogen sulfide when the loss of activity is caused by the combined action of ferricyanide and cupric ions; (d) the inactivation of  $\beta$ -amylase by aryl-mercuric compounds and disappearance of the nitroprusside reaction for free  $-SH$  groups was completely or largely reversed by subsequent treatment with hydrogen sulfide or with cysteine; and (e) irreversible inactivation of  $\beta$ -amylase by iodoacetamide which is known to enter into irreversible combination with  $-SH$  groups. This combination, as expected, was not reversed by cysteine.

a. **Antibody Against Malt Amylase.** Lüers and Albrecht (1926) carried out their experiments taking into consideration the objections raised by Bayliss regarding the existence of anti-enzyme antibodies. The question of  $H^+$  concentration, and the question of adsorption of the enzyme on non-specific precipitates were in particular studied. The reaction systems used were well buffered and controlled, and therefore there was no question of changes in  $H^+$  being responsible for the abolition of amylase activity in the presence of homologous immune serum. The amylase activity studied in the presence and absence of egg-albumin-anti-egg albumin precipitates was likewise shown to be identical; therefore, there was no diminution of amylase activity as a result of adsorption of the enzyme on non-specific precipitates.

*Experimental.* Amylase was prepared from malt according to the method of Sherman and Schlesinger (1913, 1915). Various amylase preparations were studied. One part of these preparations hydrolyzed a 3 per cent solution of starch producing 250 to 380 times as much maltose as the weight of the enzyme used.

Acetate buffer of pH 5 (16.4 ml. of 0.5 N acetate buffer in 100 ml. reaction mixture) was used to regulate the  $H^+$  concentration. In all the experiments, the amount of amylase used was from 0.0025 g. to 0.005 g. of amylase by dry weight which was capable of hydrolyzing 100 ml. of 3 per cent starch solution at pH 5 and  $20^\circ C$ . during a period of 30 minutes. To determine the degree of hydrolysis of starch, the cuprous oxide resulting from the reduction of copper reagent by

maltose was dissolved in ferric-sulfate-sulfuric acid solution and the resulting ferrous ion titrated by standard permanganate solution.

The calculation of the enzyme activity was based on reaction kinetics according to the equation characteristic of a reaction of the first order:

$$K = -\frac{1}{t} \ln \frac{a}{a-x}$$

$t$ =time in minutes;  $a$ =the concentration of starch at the start of the reaction;  $a-x$ =the concentration of starch after  $t$  minutes.

The observations of numerous investigators have repeatedly shown that after 70 to 80 per cent hydrolysis of the starch the reaction comes to an end, and from that point on the rate of hydrolysis is negligible.

*The Immunization of Rabbits with Amylase.* The rabbits used for immunization weighed 2.7 and 3.9 kg. As in immunizations with toxins, at the beginning 1 mg. amylase was given, subsequent injections being gradually increased up to 40 mg. per dose. Saline solutions of amylase were saturated with toluol to maintain aseptic conditions. None of the rabbits showed pathological symptoms. The injections were repeated every four to six, or every four to seven days. The period of active immunization was three to eight weeks, during which period a total of as much as 250 mg. of amylase per rabbit was used.

*Determination of the Anti-Amylase Content of the Immune Sera.* The amylolytic, as well as the anti-amylolytic activity of normal sera were tested and found within the range of experimental error. The mixture of immune serum and amylase one minute after mixing was added to the starch-buffer solution. They found that the decrease of amylolytic activity of such a mixture was directly proportional to the volume of immune serum added.

The inhibition of the amylase activity by the homologous immune serum was independent of the formation of a precipitate *in vitro*.

The extent of combination between amylase and anti-amylase was found to be dependent on the duration of interaction between the two reactants and on their concentrations.

The results of the experiments in regard to the inhibition of amylase by its homologous immune serum, expressed in the form of curves, showed that increase of the concentration of immune serum proportionally retards the speed of the reaction of the starch-amylase system.



In experiments 10 to 17 where the reaction mixtures were not incubated, 13.44 to 80.95 per cent of the added amylase was inhibited by the addition of 0.5 to 4 ml. of immune serum. In experiments in which the reaction mixtures were incubated, 30.6 to 95.7 per cent of the added amylase was inhibited with 0.5 to 3 ml. of immune serum.

*Inactivated Amylase as Antigen.* The enzyme was inactivated in a water bath at 56 to 58°C. for one hour. A rabbit was subcutaneously injected with increasing doses of 20 to 60 mg. of inactivated amylase during a period of one month and finally a single dose of 250 mg. was introduced. No harmful effects were observed. The author concluded that the inactivated enzyme did not produce an anti-amylase antibody.

*Specificity of Amylase-Anti-Amylase Reaction.* The highly specific character of anti-amylase prepared with malt amylase was demonstrated by the absence of any inhibitory effect on the activity of pancreatic and salivary amylases. As the optimal activity of these two  $\alpha$ -amylases are obtained at a more alkaline pH than with  $\beta$ -amylase, in the tests with pancreatic amylase the  $H^+$  concentration was adjusted to  $2.0 \times 10^{-8}$ , and in tests with salivary amylase a  $H^+$  concentration of  $5.0 \times 10^{-7}$  was maintained with phosphate buffer.

*Specificity of Amylase in the Presence of Other Antigen-Immune Serum Precipitation System.* To meet one of the objections of Bayliss that the decrease of the activity of an enzyme in the presence of homologous immune serum is due to the adsorption of the enzyme on coexisting antigen-antibody precipitate, the following experiment was carried out by Lüers and Albrecht. To 2.5 ml. of 0.001 per cent amylase solution increasing volumes of freshly obtained anti-egg albumin serum were added; after treating with the appropriate amounts of egg albumin as homologous antigen the mixture was incubated for 15 minutes at 37°C. To this was added buffered and quantitated volumes of starch solution. By varying the volumes of serum and antigen they endeavored to create favorable conditions to cause the adsorption of amylase on the precipitates and thereby to simulate the conditions Bayliss described. In none of the numerous experiments was a decrease in the activity of the added amylase in the presence of egg albumin-anti-egg albumin precipitates observed. In comparing their findings with those of earlier investigators, namely, Schütze and Braun (1907) and Braun and Schütze (1909), and Preti (1907) who reported the preparation of anti-amylase immune



sera capable of inhibiting amylase activity, Lüers and Albrecht found perfect agreement in their results and any doubt in regard to the existence of anti-enzyme antibody was removed.

### 3. Invertase and Its Properties

Five highly purified invertase preparations were obtained by Adams and Hudson (1943). All behaved as ampholytes, precipitating either with base precipitants (picric and flavianic acids, Reinecke salt, ammonium rhodanilate and picrolonic acid) or acid precipitants (cupric, or uranyl acetate). They consisted chiefly of protein although they still contained a small amount of carbohydrate. The relative activities of these preparations, at pH conditions optimal for each substrate, were found (Adams, Richtmyer and Hudson, 1943) to be in the order of

sucrose > raffinose > stachyose > inulin

Their findings agreed with the observation of Weidenhagen that one enzyme,  $\beta$ -fructofuranosidase ( $\beta$ -h-fructosidase by Weidenhagen) is responsible for the hydrolysis of the simple  $\beta$ -fructofuranosides such as sucrose and raffinose (pH 5.0–5.5), and stachyose (pH 5.1), a tetrasaccharide. They disagree with him in that inulase is another enzyme. They consider the question open whether the linkages in inulin are  $\alpha$ - or  $\beta$ -.

Despite the high concentration of  $\beta$ -fructofuranosidase in their invertase preparations no appreciable hydrolysis of melezitose (3- $\alpha$ -D-glucopyranosido- $\beta$ -D-fructofuranosido- $\alpha$ -D-glucopyranoside, or sucrose with an additional glucose molecule as a substituent group on the third carbon atom of the fructose moiety) was observed at a pH of 4.0, 5.3 or 7.0. As little as 1.5 per cent hydrolysis was detected with a large sample of enzyme. The inability of invertase to hydrolyze melezitose was attributed to the presence of the glucosido substituent on the third carbon atom of the fructofuranose moiety.

In agreement with Weidenhagen's theory, Adams *et al.* found that Baker's A enzyme preparation was *inactive* toward melibiose (6- $\alpha$ -D-galactopyranosido-D-glucose), the  $\alpha$ -phenyl- $\alpha$ -methyl-D-galactosides and  $\beta$ -methyl-L-arabinoside (at pH 4.0, 5.3 and 7.0). The presence of melibiase ( $\alpha$ -D-galactosidase) had never been reported in top yeast, so the observed inactivity was not unexpected. They found that brewer's yeast enzyme preparations containing  $\alpha$ -D-galactosidase, in agreement

with Weidenhagen's theory hydrolyzed melibiose and also the above mentioned  $\alpha$ -D-galactosides.  $\beta$ -Methyl-L-arabinoside ( $[\alpha]^{20}_D + 236^\circ$ ) which has the same configuration as  $\alpha$ -methyl-D-galactoside ( $[\alpha]^{20}_D + 196^\circ$ ) was likewise hydrolyzed by  $\alpha$ -D-galactosidase.  $\beta$ -D-galactosidase had no effect on the  $\alpha$ -D-galactosides.

The purest invertase preparation, from brewer's yeast, contained a small amount of  $\beta$ -D-glucosidase which was capable of hydrolyzing amygdalin ( $\beta$ -[d-mandelic nitrile]-gentibioside, gentiobiose ( $\beta$ -glucosido-6-glucose) and  $\beta$ -phenyl-D-glucoside. But this enzyme was not capable of hydrolyzing cellobiose ( $\beta$ -glucosido-4-glucose) or lactose (4- $\beta$ -D-galactopyranosido-D-glucose).

Invertase preparations from both brewer's and baker's yeast contained small amounts of a new enzyme, a  $\beta$ -D-mannosidase, which hydrolyzed  $\beta$ -phenyl-D-mannoside.

No evidence was obtained to indicate the hydrolysis by the purified invertase preparations of an  $\alpha$ -D-fructofuranoside (isosucrose) or of any  $\beta$ -D-galactoside,  $\alpha$ -D-glucoside (including the  $\alpha$ - and  $\beta$ - dextrins), or  $\alpha$ -D-mannoside. Melizitose and  $\alpha$ -methyl-D-manno-D-gala-heptoside also were not hydrolyzed.

The above findings are in substantial agreement with Weidenhagen's theory of the specificity of carbohydrases (p. 151).

*Certain Properties of Invertase.* The lability of invertase increases with its degree of purity. Invertase has a molecular weight of about 20,000, an isoelectric point of pH 5.0. It is inactivated in alkaline medium. In solution it is most stable at pH 4 to 5. It is reported that air dried yeast may be heated in vacuo to  $140^\circ$  to  $150^\circ\text{C}$ . without destroying all of the invertase.

The kinetics appear to be independent of the degree of purity and the condition of invertase preparations. They are the same for the enzyme in the living cell as for a solution of the enzyme. The molecules of invertase are equally active in free and in an adsorbed form (Neuberg, 1946; Griffin and Nelson, 1916; Michaelis, 1921).

**a. Antibody Against Invertase.** Schubert (1933), in the laboratory of Prof. J. M. Nelson of Columbia University, studied the controversial question of whether or not invertase is a protein.

Invertase was prepared by extracting the autolyzed yeast (*Saccharomyces cerevisiae*) with water and purified by adsorption on kaolin and elution. It manifested characteristic protein properties.

Twelve rabbits were immunized. All but one of the immunized rabbits contained anti-invertase antibody in their sera.

To determine the retardation of invertase action by immune serum initial velocities were measured (the velocity of invertase during the first 10 per cent of the hydrolysis of sucrose being constant as measured experimentally). The region ranging from pH 3 to 6.9 was investigated and the optimal retardation of hydrolysis of glucose in invertase-immune serum systems was found to occur at pH 6.5 to 6.9.

Normal serum did not affect the velocity of invertase hydrolysis of sucrose. Retardation of the hydrolysis of sucrose by the invertase-anti-invertase system was the same, whether it was measured immediately after they were mixed or after incubating for 12 to 24 hours. When the initial velocity of hydrolysis was measured by allowing the immune serum to act on the particular preparation of invertase used for immunization the retardation was 36.4 per cent. The inhibitions by the other sera ranged from 16 to 36.4 per cent.

The experiments on the specificity of anti-yeast invertase showed that the immune sera against yeast invertase had no effect on honey or taka invertase, even though yeast and taka invertases are fructosidases of the same type, which showed that the two proteins differed antigenically.

In precipitation tests the enzyme reacted with immune sera in dilutions up to 1:20,000 to 1:25,000. In hydrolysis experiments the enzyme-serum mixtures contained 0.5 ml. of serum, and 9.5 ml. of the 400-fold diluted enzyme used for immunization and tested for the precipitin reaction. In these tests the solid content of the enzyme was 1:400,000, which is well outside the limits of the precipitin reaction. Yet retardation took place in this dilution of enzyme antigen.

#### 4. Enzymatic Synthesis of Serologically and Physiologically Active Polysaccharides

The presence of serologically reactive polysaccharides in microorganisms is a well-known fact. Of the most widely studied bacterial polysaccharides those of pneumococci have been the subject of considerable interest (Heidelberger and Landsteiner, 1923; Avery and Goebel, 1933; Sevag, 1934; Goebel, 1936; Hotchkiss and Goebel, 1937; Goebel, *et al.*, 1939; Brown, 1939; Heidelberger, *et al.*, 1942, 1946; Ivánovics,

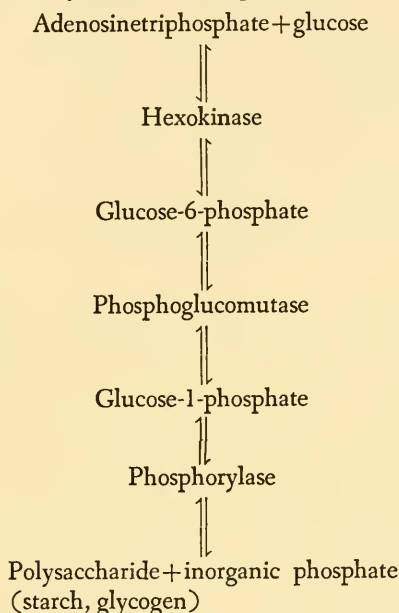
1940; Reeves and Goebel, 1941; Stacey, 1946; Evans & Hibbert, 1946). These polysaccharides as components of conjugated antigens are responsible for the serological type-specificities of forty or more types of pneumococci. The composition of each polysaccharide is unique for each type. Cross reactions among certain pneumococcal types would indicate structural relationship of their polysaccharides.

Polysaccharide of type 1 pneumococcus has been found to contain D-galacturonic acid, amino sugar and acetyl residues; type 2, glucose, aldobionic acid and uronic anhydride; type 3, cellobiuronic acid units, and; type 4, D-glucose and N-acetyl-hexosamine. The species specific polysaccharide "C" of the rough pneumococcus has been found to contain N-acetyl-D-glucosamine, a hexose and phosphoric acid. It has further been shown that type 14 polysaccharide possesses chemical and serological relationship to the polysaccharides of human erythrocytes of types A, B, AB and O (Goebel, *et al.*, 1939). Blood group A carbohydrate has been shown (Bray, *et al.*, 1944) to contain units of D-mannose, D-galactose, N-acetyl-D-glucosamine, and L-fucose.

It has been suggested that polysaccharides are integral components of physiologically active animal proteins such as hormones (Gurin, 1942, 1944; Stacey, 1946). For maximal hormonal activity the intactness of polysaccharide and protein fragments are held necessary. Gurin (1942) reported that the luteinizing and follicle-stimulating hormones prepared from the pituitary gland contain mannose and hexosamine in equimolar proportions. The gonadotropins of pregnant mare serum and human pregnancy urine appear to contain galactose rather than mannose. In these preparations the molar ratio of hexose to hexosamine was 2:1.

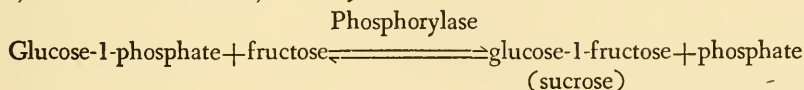
There is practically no information concerning the mechanism of the *in vivo* synthesis of above cited bacterial and animal carbohydrates. However, considerable advances have been made during recent years in the understanding of certain phases of *in vitro* enzymatic synthesis of mammalian (blood group polysaccharide), plant and certain bacterial polysaccharides. Of these, only the plant or mammalian starches, glycogen have been shown not to possess immunological properties. This is understandable in view of the fact that there does not appear to exist any configurational or structural difference among the starches and glycogens from various sources, and therefore they are not foreign to the animal host used for immunization purposes.

The processes involved in the synthesis of starch and glycogen from glucose is represented by the following scheme:



The conversion of glucose-1-phosphate to polysaccharide does not take place in the presence of highly purified phosphorylase (muscle or potato). The presence of an amount of starch, or glycogen, or dextrin as a priming or activating agent is necessary. Only the branched fraction of natural starch (amylopectin) possesses activating power. This catalytic polysaccharide is pictured as a central nucleus with side chains which are lengthened by the addition of glucose units through repetition. Linear polysaccharides have been found to lack the activating ability in the synthesis of this polysaccharide (Cori, 1945; Cori *et al.* 1945; Hassid, 1945).

Synthesis of sucrose from glucose-1-phosphate and fructose by a phosphorylase prepared from *P. saccharophila* has been reported (Hassid *et al.* 1944; Doudoroff, 1943) (For the synthesis of non-reducing  $\alpha$ -D-glucosido- $\beta$ -L-ketoarabinoside, and reducing 3-( $\alpha$ -D-glucosido)-L-arabinose, Doudoroff, Hassid and Barker, 1947a; Doudoroff, Barker and Hassid, 1947b).

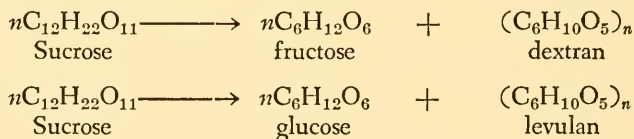




The enzyme is quite specific with respect to substrates, and causes no detectable phosphorolysis of starch, maltose, lactose, trehalose, or raffinose. Fructose cannot be replaced by fructose-6-phosphate, fructose-1,6-diphosphate, etc. However, two ketoses, 1-sorbose and d-ketoxylase have been shown to react with glucose-1-phosphate under the influence of the phosphorylase to form two new analogues ( $\alpha$ -D-glucosido- $\alpha$ -L-sorbose and  $\alpha$ -D-glucosido- $\beta$ -D-ketoxylase) of sucrose, unknown in nature (Doudoroff, *et al.*, 1944). Neither of these two analogues of sucrose was found to be susceptible to hydrolysis with yeast invertase.

*Serologically Active Dextran and Levulan.* The synthesis of polysaccharides not involving the participation of phosphorolytic reactions is that of the synthesis of dextran or levulan from sucrose. Dextran, which is a polymer of glucose in which the hexose units are joined through 1,6-linkages, is produced in large quantities by *L. mesenteroides* when the latter is grown with sucrose, but not with invert sugar (Beijerinck, 1912; Hassid *et al.*, 1940). Levulan, a fructose polymer having the 2,6-linkage has been known to be synthesized by many species of bacteria.

Cell-free enzyme preparations made from *L. mesenteroides* (Hehre, 1942; Hehre, *et al.* 1943; Hestrin and Avinieri-Shapiro, 1944) have been shown to catalyze the synthesis of both dextran and levulan without requiring the participation of organic or inorganic phosphate.



The mechanism of the synthesis of dextran or levulan from sucrose appears to be the exchange of an already existing glycosidic linkage for a new glycosidic linkage. Failure of the enzyme to catalyze the synthesis of dextran from glucose, or fructose, or a mixture of glucose and fructose, or from glucose-1-phosphate, fructose-6-phosphate, and failure of yeast inulase added to levan-sucrase to induce levulan production from inulin indicates (Leibowitz and Hestrin, 1945) that the polymerization of dextran or levulan from sucrose proceeds only on the sucrose grouping, without necessitating primary hydrolytic cleavage of the disaccharide.

*Serological Specificity of Dextran and Levulan.* Hehre (1941) reported that cell-free enzyme preparations incubated with lactose, maltose, arabinose, xylose, galactose, fructose, dextrose and a mixture of dextrose and fructose failed to produce serologically reactive polysaccharide. Sucrose, and raffinose (sucrose grouping linked with  $\alpha$ -galactose) to a slight degree, were the specific substrates for the synthesis of serologically active dextran. This polysaccharide was found to react with the antiserum of types 2 and 20 pneumococci as well as with the antiserum of the homologous bacterium, *L. mesenteroides*.

Optimal activity of the enzyme was at pH 5.0 to 6.0 and 23°C. (Hehre and Sugg, 1942; Hehre, 1946). The enzyme was completely inactivated by heating for five minutes at 55°C. The polysaccharide, unlike starch or glycogen, gave no color with iodine. It did not contain galactose, fructose, pentoses or uronic acids. On hydrolysis it contained 90 to 94 per cent reducing sugar, glucose-phenylosazone was isolated, and on oxidation with nitric acid, potassium acid saccharate was obtained.

Purified polysaccharide prepared with cell-free enzyme in 1 to 1,000,000 dilution reacted with homologous immune serum and with immune sera against pneumococcal types 2, 20 and 12. Absorption with leuconostoc cells grown in sucrose broth removed from all reactive antisera the capacity to react with dextran of culture source. When bacteria were grown in glucose broth they failed to absorb the specific antibody. Similarly, absorption with pneumococci removed the dextran reacting capacity from homologous antipneumococcal sera.

Enzyme-sucrose mixtures of strains B.M. and O, like the culture fluids of those strains, had only slight capacity to react with type 12 antipneumococcal sera in comparison to their capacity to react with types 2 and 20 antipneumococcal sera; whereas the enzyme-sucrose mixtures of strains B.C. and K, like the culture fluids of those particular strains, had as great a capacity to react with the type 12 as with types 2 and 20 antisera.

Hehre (1945) reported that enzyme preparations from *Bacillus* N9 of plant origin and *Streptococcus salivarius* isolated from a human throat catalyzed the synthesis of levulan polysaccharide from sucrose. These were found to be serologically active (Hehre, *et al.* 1944). These two different bacteria yielded identical polysaccharides. They were laevo-rotatory before and after acid hydrolysis, and yielded 96 to 97 per

cent reducing sugar. They reacted specifically with anti-rabbit sera prepared against these two organisms which had been grown in sucrose, and failed, except with some of the type 20 antipneumococcal sera, to react with other sera including those capable of reacting with dextran polysaccharide.

The purified levulan of *Streptococcus salivarius* was serologically reactive in 1 to 500,000, and that of *Bacillus* N9 in 1 to 2,000,000 dilution. Immunization of rabbits with suspensions of these bacteria which had grown in sucrose media regularly yielded levulan reactive immune sera, whereas immunizations with suspensions of the same species of bacteria grown in glucose media regularly yielded antisera which were entirely non-reactive with levulans although they did agglutinate in high dilutions suspensions of the bacteria which had been used for immunization.

## 5. Antibody Against Bacterial Polysaccharidases

Sickles and Shaw (1950) obtained antisera in the rabbits against the polysaccharidases isolated from various strains of *B. palustris*. These enzymes decomposed the capsular polysaccharides of pneumococci Types III and VII. The enzyme which specifically decomposed Type III polysaccharide was neutralized by homologous anti-enzyme immune sera and none of these antisera were specifically active against Type VII polysaccharidase and *vice versa*.

In mouse protection tests corresponding to the neutralization tests *in vitro*, the results were comparable. The results showed that the neutralizing activities of immune sera were directed specifically against the polysaccharidases.

## 6. Antibody Against Crystalline Lysozyme

Lysozyme is an enzyme which lyses *Micrococcus lysodeikticus*, and *Sarcina lutea*. According to Meyer and Hahnel (1946) the substrate is a mucopolysaccharide, which is rapidly depolymerized by lysozyme. Optimal conditions for the antibacterial action of lysozyme on *M. lysodeikticus* is said to parallel in general the conditions for its depolymerizing action (Feiner, Meyer, and Steinberg, 1946). The polysaccharide isolated from *M. lysodeikticus* is precipitable by antibacterial serum and is one of the antigenic components of the organism. Feiner, *et al.*

(1946) reported that lysozyme (from egg white) is capable of attacking organism or substrate when either is combined with antibody. Apparently the reaction between the antibacterial serum and the antigen does not block the lysozyme-susceptible groupings of the polysaccharide.

Smolens and Charney (1947) reported the production of antibody in rabbits against four to six times crystallized egg white lysozyme. This enzyme has a molecular weight of 15,000 to 18,000. Antibody to lysozyme was produced in some rabbits and not in others. Lysozyme immunologically is species specific.

To varying concentrations of crystalline lysozyme varying dilutions of immune serum were added and incubated at 37°C. for one hour. Then to each mixture a certain volume of *M. lysodeikticus* suspension was added and incubated at 37°C. and lysis was observed at various intervals of five-hour periods. Under these conditions the lytic activity of the enzyme was blocked.

## D. HYALURONIDASE OR THE PERMEABILITY FACTOR

### 1. Discovery of the Permeability Factor

Duran-Reynals (1928, 1929) discovered that testicular extracts exercised a virus enhancing property. McClean (1930) confirmed the observation of Duran-Reynals and ascribed the effect of testicular extracts to an immediate increase in tissue permeability. The increase in dermal permeability was shown by the rapid disappearance of the bleb produced by the intracutaneous injection of these extracts and by rapid spread through a large area of skin of any suitable colored indicator that was injected simultaneously.

McClean (1931) also stated that testicular extract causes swelling and distortion of the fibre bundles of the dermis. According to Duran-Reynals (1933; see also Hoffmann and Duran-Reynals, 1931) the outstanding properties of the factor derived from tissue are: It enhances the infectivity of all bacteria and viruses so far tested; it increases tissue permeability, as shown by the spread of injected material, and it possibly increases cell permeabilities as shown by experiments on red blood cells and sea urchin eggs. The factor from one species will enhance infections not only in the same species, but in all other species susceptible to the infectious agents. Intravenous injection of



testicle extract induces a general increase in permeability of the skin with a correspondingly increased susceptibility of the skin to infectious agents.

The Duran-Reynals effect was observed on the enhancement of staphylococcal infection by the presence of testicular extract with the infective dose (Duran-Reynals and Suner-Pi, 1928). Pijoan (1931) found that the extracts from testicle, kidney, and spleen enhanced the infective power of 20 strains of various bacteria to a high degree. Hoffmann (1931) reported that this factor promoted the pathogenic action of the viruses of herpes, vesicular stomatitis of horses, Borna disease, and vaccinia. Favilli (1931) reported that testicle extract caused notable fragility of red blood cells *in vitro*. It was reported that this factor obtained from the testicles of rat, rabbit and bull prevents or retards the growth of a rabbit tumour when a mixture of the extract and a tumour cell suspension is inoculated intradermally (Duran-Reynals, 1931). Boyland and McClean (1935) reported that aqueous extracts of rapidly growing grafted mammalian tumours contain a factor which, on intracutaneous injection into the rabbit, increases the permeability of the dermis. The rate of the growth of the tumour was stated to be a measure of the amount of the factor present. Duran-Reynals (1933), pointed out that this factor is present in the filtrates of virulent strains of pneumococci and streptococci.

Following the demonstration by Duran-Reynals that bacterial filtrates contain a factor similar to that extracted from testicles, McClean (1936) investigated the filtrates of *Cl. welchii*, *Cl. paludis*, *Cl. ovitoxicus*, *Cl. oedematis-maligni*, *Cl. oedematiens* and *Cl. tetani*. Of these, the most powerful was that of *Cl. welchii*. He found that 0.000,015 ml. of the original toxin filtrate of *Cl. welchii*—equivalent to 0.0014 of the M.L.D. for a mouse—produced immediate diffusion in the dermis, and a sensible increase in an area of diphtheria toxin lesion as compared with the controls. The results with the filtrates of the other organisms appeared to show that the diffusing activity of these filtrates does correspond approximately to the relative local invasiveness of the organisms from which they were obtained.

The dry weight of the smallest dose of the purified diffusing factor of *Cl. welchii* was 0.000,000,5 mg., or  $5 \times 10^{-4}$  microgram, which was found to correspond to the activity of the purified testicular extracts.

The diffusing activity was stated not to be particularly related to



any of the various toxins produced by different types of *Cl. welchii*. No increased diffusion was caused by two samples of *Cl. histolyticum* toxin or by cultures of *Cl. tetani*.

## 2. Enzymic Nature of the Permeability Factor and the Nature of the Substrate

Hoffmann and Duran-Reynals (1931) reported that the permeability factor was destroyed by heating at 60°C. for 30 minutes, and also that  $5 \times 10^{-4}$  microgram represented an effective permeability dose (McClean, 1936). These facts would suggest that the substance acts as an enzyme.

Meyer and Palmer (1936) reported that vitreous humor and umbilical cord contain a mucopolysaccharide consisting of N-acetylglucosamine and glucuronic acid in equimolecular concentrations. A chemically similar and serologically inactive mucopolysaccharide was likewise isolated from the mucoid phase of Group A hemolytic streptococci (Kendall, Heidelberger and Dawson, 1939), from synovial fluid (Meyer, Smyth and Dawson, 1939), and from fowl sarcoma\* (Kabat, 1939). This carbohydrate was named hyaluronic acid by its discoverers.

Meyer, Dubos and Smyth (1937) reported that the autolytic system of pneumococcus hydrolyzes the mucopolysaccharide from vitreous humour, umbilical cord and streptococcus. This enzyme was regarded as being the same as that responsible for the autolysis of heat-killed pneumococci.

It was left to Chain and Duthie (1939, 1940) to bridge the link between the enzyme hydrolyzing the mucopolysaccharide and the spreading factor. They found that the testis extract is active as a spreading factor in a dilution of  $10^{-8}$ . It contained a proteolytic enzyme, but this was not responsible for the permeability effect as crystalline trypsin showed no similar effect. The enzyme present in the testicular extract reduced the viscosity of synovial polysaccharide to 1/300 of its original value (approximately that of water). Its action also caused the hydrolysis of the polysaccharide, yielding N-acetylglucosamine and glucuronic acid, which is the same as found by Meyer, *et al.* The testicular extract produced the same enzymatic effect

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\*Pirie (1942) isolated a viscous polysaccharide from two fowl tumors that did not contain hyaluronidase.

on the polysaccharide of vitreous humor. They called this enzyme mucinase, and considered it identical with the spreading factor.

In the meantime, Meyer, *et al.* (1940a and b), described the nature of the pneumococcal enzyme hydrolyzing hyaluronic acids from various sources. This enzyme was also prepared from Group A hemolytic streptococci, though the activity was somewhat less than that of pneumococci. The latter acting on hyaluronic acid caused a fall of viscosity, and its hydrolysis into N-acetylglucosamine and glucuronic acid. They called this enzyme "hyaluronidase" (1940b). The action of pneumococcal enzyme on the hyaluronic acids from various animal, bacterial and tumour sources was specific. It acted also on a synthetic hyaluronic acid trisulfuric ester. On the other hand, an enzyme preparation from *Cl. welchii* reacted less specifically. It hydrolyzed starch, glycogen, neutral polysaccharide, chondroitin-sulfuric acid, and mucoitin-sulfuric acid from pig gastric mucosa, in addition to hydrolyzing natural hyaluronic acid. Evidently it represented a mixture of enzymes. They also stated that the pneumococcal enzyme acting on hyaluronic acid is not the same as that responsible for the lysis of pneumococci.

About the same time, Chain and Duthie (1940) published their second paper in which they dropped the name mucinase and adopted "hyaluronidase." In a paper, appearing some months before that of the above authors, Robertson, Ropes and Bauer (1940) used the term mucinase for the mucolytic enzyme they isolated from *Cl. perfringens*. Chain and Duthie (1940) measured the activity of hyaluronidase by the amount of reducing substances and N-acetylglucosamine it liberated, and the rate of the fall of the viscosity of hyaluronic acid. The enzymatically active solution was likewise tested for its spreading property. In a series of nine animals, they found that in every case a clearly recognizable difference between the area of spread was produced by 0.25 and 0.1 units of the standard testis preparation. Comparing the unit activity of the spreading factor of standard testis extract, leech extract (Claude, 1937, 1940), copperhead venom, black tiger venom, bee sting, *Cl. welchii* toxin and pneumococcus culture with the hyaluronidase activity, good agreement was obtained with testis, leech and venom enzyme. With bacteria and snake venom hyaluronidase, somewhat bigger spreads were obtained than were expected from their hyaluronidase content. The reason for this difference, they believed, was most probably due to the oedema caused by these fluids.

They isolated, as substantiating evidence, a substance closely resembling hyaluronic acid from the skin of rabbits. When acted upon by testicular hyaluronidase, the skin hyaluronic acid was hydrolyzed in the same way as the hyaluronic acids isolated from other sources. Salivary, gastric and duodenal mucin, and mucin of the uterine cervix were not hydrolyzed by hyaluronidase.

Robertson, *et al.* (1940), experimenting with a purified enzyme (mucinase) from *Cl. perfringens (welchii)* arrived practically at the same conclusion that Chain and Duthie had reached regarding the nature and properties of hyaluronidase. Their enzyme preparation was 900-fold more active than the original filtrate. In contrast to the findings of Meyer, *et al.*, however, and in agreement with Chain and Duthie, their enzyme preparation had no effect on human or swine gastric mucin, salivary mucin (human) or the chondroitin-sulfuric acid obtained from cartilage. Viscous solutions of mucins obtained from umbilical cord (human) and loose abdominal connective tissue fascia (rabbit) rapidly lost their viscosity and characteristic precipitability by the enzyme.

The enzyme prepared by Robertson *et al.*, from autolytic pneumococci was compared with the enzyme from *welchii* as follows:

TABLE IX

Tests	Mucinase	Autolytic enzyme of pneumococcus
Lysis of pneumococci	—	+
Effect of alcohol or acetone	none	inactivates
Effect of iodine	irreversibly inactivates	reversibly inactivates
Effect of arsenite	inactivates	reactivates
Effect of cyanide	inactivates	none
Hydrogen peroxide	none	inactivates
pH at optimal activity	3.9–8.5	4.5–8.0

The data show that the autolytic enzyme of pneumococcus is not the same as hyaluronidase (mucinase).

The correlation of the mucolytic activity of hyaluronidase (mucinase) with the spreading property has been confirmed also by the reports of Favilli (1940). He confirmed the observation of Duran-Reynals (1939) that snake venom possesses spreading factor, and further found that venoms hydrolyze mucopolysaccharides.

McClean and Hale (1940, 1941) reported a large amount of confirmatory experimental data regarding the various properties of hyaluronidase, such as described above. They prepared purified enzymes from testicular extracts, *Cl. welchii*, and *Cl. oedematis-maligni* (vibron septique), and experimented with dried snake venom. These preparations in high dilutions caused rapid diffusion in the skin, and showed marked hyaluronidase activity as demonstrated by the reduction of viscosity and the liberation of N-acetylglucosamine. The optimal activity was at pH 4.6 which was also the pH of optimal activity for the enzyme from all the sources examined. The activity for the enzyme derived from bacteria falls below pH 4.6, and is slow above pH 4.6 up to 8.5. Viper venom enzyme was completely inactive above pH 6.5.

The testicular enzyme did not reduce the viscosity of any of the starch pastes (rice, maize, potato and wheat), but purified enzymes derived from *Cl. welchii* and *vibron septique* reduced the viscosity of both potato and wheat starch. The rate of the reaction bore no relation to their hyaluronidase activities. No Benedict reaction could be obtained after 24 hr. incubation of starches with bacterial enzymes, notwithstanding the substantial fall in viscosity that had occurred; the iodine reaction also remained positive. Takadiastase, which contains no hyaluronidase and does not diffuse in the skin, caused a rapid fall in the viscosity of starch paste with the liberation of reducing sugars. These findings showed that hyaluronidase has no amylase activity.

Hyaluronidase was found to have no action on disodium phenylphosphate (for phosphatase) and diphenylphosphate (for phosphodiesterase).

The enzymes either had no, or very slight, action on gastric mucin, with the exception of those derived from *Cl. welchii* and *vibron septique* which liberated reducing substances from gastric mucin. This confirms in part the observations of Meyer, *et al.*, and contradicts those of Robertson, *et al.*

The question of whether or not heparin, closely allied to hyaluronic acid in chemical structure, is attacked by hyaluronidase was investigated. It was found that viper venom, testicular or *Cl. welchii* enzymes exercised no action on heparin; they do not, therefore, act in a manner comparable with thrombokinase.



Madinaveita and Stacey (1944) studied the effect of testicular hyaluronidase on carbohydrates from various sources. It acted on hyaluronic acid, closely related polysaccharides from placenta and tumor mucin and lowered the viscosity of chondroitin sulfate from nasal septa. The enzyme showed no activity when tested with the following polysaccharides as substrates: Blood Group A polysaccharides from both gastric mucin and from pepsin, the latter containing units of l-fucose, mannose, galactose and N-acetylglucosamine; a galactan from the pancreas; ovomucoid, containing units of N-acetylglucosamine, mannose and galactose united in a complicated branched chain structure; dextran from *L. dextranicum* which contains long chains of glucose units linked through the 1- and 6- positions; the *Rhizobium radicicolum* polysaccharide containing units of glucuronic acid and glucose linked in a manner generally similar to type III pneumococcus polysaccharide; luteose from *P. luteum* Zukal, a 'β-dextran' constituted solely of units of glucose linked through the 1- and 6- positions; and *Azotobacter* polysaccharide containing units of glucuronic acid and glucose; and the *B. megatherium* levan which is built up from units of fructose linked through the 2- and 6- positions.

### 3. Mechanism of the Action of Hyaluronidase

Despite intensive studies carried out during the last decade, hyaluronidases obtained from different sources are still considered mixtures of enzymes. Their specific hydrolytic action still is not clearly defined. According to Rogers (1946), hyaluronidases of *Cl. welchii*, streptococci and bull testes liberate from hyaluronate units of different average sizes with various reducing capacities. Streptococcal enzyme, for example, releases freely diffusible reducing sugar and leaves no reducing polysaccharides, whereas testicular hyaluronidase leaves non-diffusible units which account for as much as 20 per cent of the liberated reducing sugar. Hahn (1945, 1946) reported that at pH 4.6 one testicular enzyme degraded hyaluronate to freely diffusible disaccharides while a second completed hydrolysis to monosaccharides. These findings indicate that each preparation of hyaluronidase is a mixture of enzymes responsible for various stages of the hydrolysis of the hyaluronate. Hahn (1945) reported likewise that at pH 4.6 *Cl. welchii* hyaluronidase preparations do not degrade the polysac-



charide to monosaccharide. At pH 7.0, however, with proper buffering and salt concentration, a large proportion of the reducing sugar liberated by this enzyme appeared to be monosaccharide. Meyer, *et al.* (1940a) had already shown that their *Cl. welchii* preparation liberated 91 per cent of the theoretical amount of the reducing sugar when acting at pH 6.0 (optimal pH 5.8) determined under their salt and buffer conditions. According to Meyer (1947), the dual nature of the two glycosidic linkages in hyaluronic acid, one belonging to the N-acetylglucosamine, the other to the glucuronic acid moiety, suggests that the depolymerization and hydrolysis into monosaccharides requires two enzymes. Thus it was observed that pneumococcal hyaluronidase hydrolyzed the substrate to 100 per cent of the theoretical amount whereas testicular hyaluronidase hydrolyzed the substrate to only 50 per cent. The addition of the pneumococcal hyaluronidase to the non-hydrolyzed residue brought about complete hydrolysis, while the addition of fresh testicular enzyme had a negligible effect.

Experimenting with testis, *Cl. welchii* and streptococcal culture filtrates, Humphrey (1946) observed that at pH 6 to 7.0 they acted in different ways upon hyaluronic acid, although all of them caused hydrolysis with the liberation of reducing substances and substances which were estimated for N-acetylglucosamine contents. When assayed under identical conditions of salt concentration and pH, testis and *Cl. welchii* enzyme preparations showed so close relationship between their power to reduce the viscosity of hyaluronic acid and to bring about its hydrolysis to small molecules that it was thought very probable that these two actions are brought about by the same mechanism. Humphrey (1946a) suggested that hyaluronic acid contains, beside glycosidic linkages between glucuronic acid and N-acetylglucosamine, preformed ring compounds involving N-acetylglucosamine, or that the enzymic hydrolytic products are peculiarly liable to form such compounds. The fact that the testis enzyme with almost negligible, and streptococcus enzymes with negligible  $\beta$ -glucosaminidase activity can hydrolyze hyaluronic acid in characteristic fashion, led Humphrey (1946b) to conclude that such activity is not necessary for hyaluronidase, and this was borne out by the failure of  $\beta$ -glucosaminidase preparations to show hyaluronidase effects.

The mechanism of the action of hyaluronidase on hyaluronic acid is still far from being clarified. However, the fact that the enzyme (testis)

exercises: (a) a powerful hydrolytic action on cartilage, chondroitin sulfuric acid, polysaccharide containing sulfuric acid and pig-skin polysaccharide containing sulfur whose hydrolysis parallels that exercised against hyaluronic acid which is free from the sulfuric acid group, and (b) no action on heparin and mucoitin sulfuric acid, indicates that the specificity of this enzyme is directly related to the specific structure of the polysaccharide moiety of the above mentioned active substrates of dissimilar composition. For a review on the chemical composition of these substrates the reader is referred to an article by Meyer (1947).

**a. Quantitative Methods of Measuring Hyaluronidase Activity.** For the measurement of the activity of hyaluronidases several methods have been employed:

*Mucin Clot Prevention Method.* Seastone (1939) found that streptococcal capsular polysaccharide when mixed with serum protein, gives a typical mucin clot in the presence of acetic acid. After the treatment of polysaccharide with hyaluronidase (mucinas), however, this reaction no longer takes place (Robertson, Ropes and Bauer, 1940). In an attempt to correlate hyaluronidase activity quantitatively with diffusing activity, McClean (1943) described a method which depends upon destruction by the enzyme of the power of a substrate-protein complex to form a typical fibrinous clot on the addition of acetic acid. After incubation with the specific enzyme the quantity of the clot is reduced and the character of the precipitate changes from a fibrous to a flocculent precipitate, yielding finally a clear solution. McClean reported that the destruction of the clotting power of the substrate appears to develop in an early stage of its degradation, this loss being detectable before any appreciable fall in viscosity occurs. The test is positive only with crude hyaluronate and when carried out in the native fluid or with the isolated and redissolved protein salt. Pure hyaluronate precipitates protein in acidic solution in flocculent form as contrasted with the fibrous clot obtained with crude hyaluronate-protein complex. Meyer (1947) failed to find a correlation between mucin clot prevention units and viscosity reducing units, or a constant trend associated with the potency or the source of the enzyme.

*Turbidimetric Method.* Kass and Seastone (1944), in their study of the relation of hyaluronidase activity and virulence of *hemolytic streptococcus*, developed a turbidimetric method of determining the activity and the concentration of the enzyme of bacterial origin. The method

is based on the ability of the enzyme to decrease the capacity of the mucoid polysaccharide to precipitate acidified (pH 4.2) protein. Two units of hyaluronidase were considered equivalent to one viscosity-reducing unit (see below). Hahnel and Meyer (Meyer, 1947) modified this method so that the ratio of the units determined viscosimetrically to those measured turbidimetrically varied only from 1.2 to 1.4.

*The Viscosity Reducing Method.* This method is based on the principle that a solution of a given molecular species flows at a certain speed. As the molecular size increases the rate of flow of the solution decreases. Splitting of the molecules by enzyme action into smaller molecular units increases the rate of flow of the resultant solution. The concentration of the solution, the presence of salts, buffer, and temperature exercise controlling effects on the rate of flow of a given solution. Madinaveitia and Quibell (1940) made use of viscosimetric measurements to study the relative activities and degree of purity of various hyaluronidase preparations. Measuring the time required to reach the half-life of the substrate, they observed that enzyme activity runs roughly parallel with the spreading activity of the enzyme in the skin. McClean and Hale (1940, 1941) made extensive use of this method in their study of hyaluronidases.

*Viscosity-Reducing-Unit, (V.R.U.),* was defined as that concentration of enzyme which reduced the viscosity of a standard substrate preparation to a level half-way between its original figure and that of the solvent employed, in 20 minutes. The selection of this period of time was determined by experimental conditions. The following units of activity were found in various enzyme preparations.

Although a half viscosity level was reached in between one and 10

TABLE X

Preparations from:	Diffusion dose in ml.	Hyaluronidase units
Testis extract	$4 \times 10^{-5}$	90.0
Viper venom	$1.6 \times 10^{-2}$	0.5
Cl. welchii	$4 \times 10^{-6}$	70.0
Cl. oedematis-maligni	$4 \times 10^{-5}$	22.0
Staphylococcal filtrate	$4 \times 10^{-2}$	60.0
Pneumococcal filtrate	$4 \times 10^{-2}$	6.0
Streptococcal filtrate	$4 \times 10^{-2}$	0.3

minutes, no measurable amount of acetylhexosamine could be detected for 50 minutes; the maximum liberation was not attained until considerably after two hr. had elapsed. When the concentrations of the enzymes from different sources were adjusted so that their reaction times for the reduction of viscosity were similar, it was found that the amounts of enzyme necessary to liberate measurable quantities of reducing substance were approximately the same whatever the source of the enzyme. The time lag in the liberation of reducing substances, coupled with the lack of complete correlation between this activity and the reduction of viscosity, suggested that two separate mechanisms may be involved; if the enzymes are not identical, they must be closely associated, since they were not separated by any existing method of purification and were similarly influenced by variations in the environment of the reaction.

#### 4. Distribution and Physiological Significance of Hyaluronidase or the Permeability Factor

All the investigators confirmed the original observation of Duran-Reynals that testicular extracts contain this enzyme or the permeability factor. It is likewise found in leech head extracts (Claude, 1937, 1940; Favilli, 1940) and in certain snake venoms (Duran-Reynals, 1939; Favilli, 1940, etc.). The findings of Duran-Reynals in this regard are as follows: The factor is most abundant in the venom of the *Viperidae* (rattlesnake) family and relatively scant in the venom of the *Colubridae proteroglypha* (cobra) family, and it is absent from toad venom. Extracts of the supralabial glands of harmless snakes contain only negligible amounts of the factor. It is present in various normal tissues (Duran-Reynals, 1928) and in rapidly growing grafted mammalian tumors (Boyland and McClean, 1935).

a. **Relation of Hyaluronidase Production to Virulence of Bacteria.** The presence and absence of this factor in numerous bacteria has been reported. Some of these reports are confirmatory and certain others contradictory. The discrepancy among the findings of various investigators may be due to differences of strains used, method of growing and the age of the culture, methods of testing the activity of the preparations, and methods of preparation of enzymes. Such differences of enzyme activities among various strains of a given type or species



of bacteria are well known. A satisfactory correlation of the invasive or virulent character of a microorganism with its ability to elaborate a certain pertinent enzyme *in vivo* would seem to require the study of the two factors under identical conditions. Attempts to correlate the invasiveness of a bacterium with its ability to produce hyaluronidase *in vitro*, no doubt, fails to meet the above criterion. The constancy of optimal pH, the presence of critical substances, the age of the culture, the rate of the autolysis of bacteria, the stability or resistance to denaturation effects, the action of proteolytic enzymes, and the presence or absence of inhibitors are factors which play a significant role on the amount and activity of a given enzyme produced during *in vitro* growth. The virulence or invasive ability of organisms harvested from such an environment cannot be assumed to have an unfailing correlation with the activity and the amount of hyaluronidase that may or may not be produced. The inclusion of the specific whole substrate in the growth medium has been reported to enhance or stimulate (or protect from destruction) certain bacterial enzymes, such as hyaluronidase. And since the ground substance of the connective tissue is a viscous substance containing, probably, hyaluronate, the *in vivo* environment may offer the ideal condition for the maximal production of hyaluronidase and, therefore, for its correlation with the invasive or virulent character of the organisms. In this respect organisms, such as staphylococci, streptococci and certain clostridia, which make use of host's skin, rich in hyaluronidase substrate, as an entrance into the body prior to spreading to other sites, would seem to offer a suitable means to correlate bacterial invasiveness with the ability to elaborate hyaluronidase at the site of initial infection. From this point of view, as discussed below, the studies of McClean, *et al.* (1943), and McClean and Rogers (1944) are most significant. We will first discuss the results of studies on the production of hyaluronidase by bacteria grown *in vitro*. These will be followed by the presentation of the results obtained in *in vivo* experiments by Kass and Seastone (1944) and McClean (1941) and McClean and his associates (1943, 1944).

Crowley (1944) tested 308 strains of group A streptococci for hyaluronidase production. Only two serological types (types 4 and 22) showed hyaluronidase activity. Of the 65 strains of groups C and G streptococci tested 48 showed hyaluronidase activity. Hyaluronidase producing strains were stated to be invariably non-capsulated. She failed



to obtain any correlation between hyaluronidase production, type of strain and virulence for man. Humphrey (1944) surveyed the hyaluronidase production by 81 strains of pneumococcus isolated from successive cases of pneumonia, and failed to find a correlation between the amount of hyaluronidase produced and the severity of the clinical infection, or between enzyme production and type. Organisms of Type I rarely produced hyaluronidase.

Kass, Lichstein and Waisbren (1945) reported that of 32 strains of *Cl. welchii*, 12 produced hyaluronidase. Of the 12, 11 were toxigenic. Of the 20 strains which were hyaluronidase negative, 11 were toxicogenic. Of twenty strains isolated from cases of gas gangrene, 18 produced hyaluronidase. Thus, only 54 per cent of the 94 virulent strains of *Cl. welchii* produced hyaluronidase. On the basis of the results reported, they concluded that regardless of the role of hyaluronidase in a gangrenous lesion, its *in vitro* production by a given strain of *Cl. welchii* bears no necessary relationship to the virulence of that strain for mice.

Schwabacher, *et al.* (1945) tested 814 strains of staphylococci and micrococci from both healthy carriers and clinical infections, the latter mostly wounds, for the production of hyaluronidase, "coagulase," and  $\alpha$ -hemolysin. Of the 654 coagulase-positive strains, 86.7 per cent were hyaluronidase- and  $\alpha$ -lysin, positive. Of the remainder, 4.4 per cent were hyaluronidase-negative and  $\alpha$ -hemolysin-negative, and 2 per cent were negative for both. They were not clear what part hyaluronidase plays in determining the virulence of a strain of *Staphylococcus aureus*. (For a review on hyaluronidase in bacterial infection see Duff, Murray and Fleming, 1946).

In connection with the production of hyaluronidase by bacteria the effect of the presence or absence of hyaluronic acid and its split products in growth media has been studied. Meyer, *et al.* (1940a, 1940b), McClean and Hale (1941), and Kass, *et al.* (1945) reported that the production of marked amounts of hyaluronidase by *Cl. welchii* was demonstrated when grown in the presence of hyaluronic acid. Rogers (1945) reported that in peptone-free and protein-free but adequately buffered media, strains of streptococci, *Cl. welchii* type A, staphylococci and *Cl. septicum* produced large amounts of hyaluronidase. However, streptococci and *Cl. welchii* produce an increased amount of hyaluronidase in proportion to the amount of hyaluronate

added to the buffered growth medium. The hydrolysis of hyaluronate by streptococcal hyaluronidase destroys this stimulative effect on these organisms, whereas the action of testicular hyaluronidase does not. Staphylococci and *Cl. septicum* do not respond to the inclusion of hyaluronate either in the simplified or in the more complex growth media. These organisms require factors present in peptone or digest media for optimal hyaluronidase production. Streptococci and *Cl. welchii* do not require such factors. Rogers (1946) also reported that potassium hyaluronate hydrolyzed by crude or purified testicular hyaluronidase can stimulate hyaluronidase production by streptococcus or *Cl. welchii* equally as well as the unhydrolyzed polysaccharide. In contrast, potassium hyaluronate hydrolyzed by streptococcal or *Cl. welchii* enzymes does not stimulate hyaluronidase production by streptococcus. The streptococcal hydrolysate does, however, stimulate hyaluronidase production by *Cl. welchii*, whilst the *Cl. welchii* hydrolysate has a small but probably significant effect on the latter organism. Hydrolysis of hyaluronate by mild treatment with acids destroys the ability of the polysaccharide to enhance hyaluronidase production by streptococcus. (For a discussion of the relation of the above observed effects to the "adaptive enzyme" concept see elsewhere in this monograph and Sevag, 1946).

**b. Relation of Capsular Polysaccharide and Hyaluronidase to the Virulence of Streptococcus and Pneumococcus.** In connection with the relation of hyaluronidase to virulence, it is significant that Kass and Seastone (1944) found a noteworthy role for the capsular polysaccharide in the virulence of Group A haemolytic streptococcus. Hyaluronidase added to a phagocytic system containing defibrinated human blood, immune or non-immune, greatly increased the rate of phagocytosis of Group A streptococci. Under the same conditions phagocytosis of Type I pneumococci was not affected by hyaluronidase. The bactericidal activity of non-immune blood against Group A streptococci was increased by hyaluronidase; the activity of immune blood was, however, somewhat inhibited by the enzyme. Mice could be protected against Group A streptococcus infection by frequent treatment with 200 turbidity-reducing units of hyaluronidase. Mice infected with type I pneumococcus and treated with hyaluronidase died somewhat sooner than the untreated controls. The consistent presence of hyaluronic acid in Group A streptococci isolated from human infections indicated

to them the likelihood of this polysaccharide serving as an armor against the host. That this defense is eliminated by the action of injected hyaluronidase in animal experiments is indicated by the above reported results.\*

**c. Hyaluronidase in Infected Tissue Extracts.** McClean, *et al.* (1943) have demonstrated that in laboratory animals hyaluronidases can be detected in the edema fluid and tissue extracts of experimentally induced infections within a few hours of the original inoculation, and suggested that this might form the basis of a rapid and accurate method of identifying the causal organisms in naturally infected wounds.

In view of a negative report by MacLennan (1944), McClean and Rogers (1944) reported the result of an extensive study. They investigated: (a) the influence of antitoxin, given after infection, on the secretion of hyaluronidase (and lecithinase) in the tissues; (b) the effect of employing very small doses of organisms to initiate infection; (c) the effect of mixed infection with proteolytic organisms on these enzymes; and (d) the synergic action of proteolytic *Clostridia* and other wound-infecting organisms. Guinea pigs were infected by intramuscular injection of washed 18-hour cultures together with calcium chloride.

Hyaluronidase was detectable at the earliest microscopic sign of infection and its titre rose steadily. Lecithinase was easily detectable in the animals infected by a 100-fold dilution and rose to the same titre as in animals receiving the undiluted inoculum. The number of organisms introduced did not influence the production of hyaluronidase once infection was established. In mixed infections with *Cl. welchii* or *septicum* together with *Cl. sporogenes* or *histolyticum*, there was no evidence of any suppression of the enzymes by the latter two proteolytic organisms as had been suggested by MacLennan (1944).

In the animals that had received antitoxin the appearance of enzymes, both in the muscles and edema fluids, was usually inhibited and sometimes suppressed. Suppression of the enzymes was not due to the failure of the organisms to multiply; for they could be seen in the stained films, there were no obvious signs of infection, and other animals given the same dose of antitoxin eventually died. On the basis

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\*McClean (1942) reported that hyaluronate partly depolymerized by precipitation with acetic acid inhibited the decapsulation of streptococci by testicular hyaluronidase. Gastric mucin also inhibited the decapsulation.

of these results they concluded that tests for hyaluronidase and lecithinase are likely to be negative in patients who have had an adequate dose of antitoxin before removal of samples. On the other hand, the presence of these enzymes in either exudate or muscle of a patient who has received antitoxin would indicate that antitoxin is urgently required.

d. **Effect of Hyaluronidase on Fertilization.** McClean and Rowlands (1942) reported that hyaluronidase was capable of liquefying the highly viscous gel which cements the cumulus cells around the unfertilized tubal egg of the rat. This finding was confirmed by Fekete and Duran-Reynals (1943) showing that extracts from rattlesnake venom, leech tissues, and testicle, known to be very rich in hyaluronidase, exercised a very pronounced effect in dispersing the follicular cells surrounding the ova of mice; this effect was stated to be an indispensable step in fertilization. Rowlands (1944) suggests that the gel is hyaluronic acid similar to that in synovial fluid and, to enable its disintegration to occur as a preliminary to fertilization, a certain unspecified concentration of hyaluronidase must be established by the presence of an adequate number of sperms in the vicinity of the egg. The test showed that although the action of hyaluronidase is quite variable under the conditions tested, it increased the fertilizing capacity on the average to the extent that treated groups required about one sixth of the sperm concentration to give a 50 per cent response, compared with that in control groups. Monroy and Ruffo (1947) reported that a substance from the sperm and testes of *Arbacia lixula* (=putulosa Auct.) and *Spoerechinus gran*, dissolves the jelly-coat of sea urchin eggs and also lowers the viscosity of mucin solution. Recent studies make, however, the role of hyaluronidase in fertilization controversial.

## 5. Non-Specific and Specific Inhibitions of Hyaluronidase

a. **Non-Specific Inhibition of Hyaluronidase.** McClean (1942) reported the inhibitory action of heparin, chondroitin sulfate and mucin on the *in vitro* decapsulation of streptococci by testicular hyaluronidase. The neutral polysaccharides of Shiga-Kruse bacilli and a blood Group A hapten failed to do so. Meyer (1947) reported that heparin desulfurated with oxalic acid-barium oxalate failed to inhibit hyaluronidase, showing the importance of acidic groups in these inhibitions.



Guerra (1946) reported that sodium salicylate inhibited the spreading effect of hyaluronidase. In a total of 96 experiments on 24 albino rabbits, he observed that the spread area of India ink with hyaluronidase was six times greater than with saline. The oral or intravenous administration of sodium salicylate inhibited by 57 to 66 per cent the spreading effect of hyaluronidase; the degree of inhibition varied with the dose of salicylate administered. He concluded that the evidence found in normal rabbits and humans, as well as in individuals who have latent or active rheumatic fever, indicates the important role of hyaluronidase in its mechanism and the inhibitory effect of sodium salicylate as a typical antirheumatic drug. Meyer (1947) reported that in *in vitro* experiments equivalent or higher concentrations of salicylate are without effect on the depolymerization or hydrolysis of hyaluronate, but the permeability of rabbit skin was depressed by the action of salicylate.

In a series of three papers, Haas (1946) reported that normal plasma contains a non-specific, highly active enzyme (anti-invasin I, using the term invasin for hyaluronidase) which rapidly destroys hyaluronidase. He reported also a proinvasin I which is found in bacteria and venoms which rapidly inactivates antinvasin I. On the other hand, normal plasma contains an antinvasin II which destroys proinvasin I, and thereby indirectly counteracts invasion. He reported a number of other enzyme factors interfering with one or the other above reactions. Dorfman, *et al.* (1947) investigated the claims of Haas and found that the reaction between hyaluronidase and antinvasin I is virtually complete in three minutes despite the presence of unreacted hyaluronidase. They suggested that this reaction is not enzymatic in nature, but that it is due to an inhibitor of protein nature (Dorfman, *et al.*, 1948). Meyer (1947) declined to agree with Haas' claims for experimental reasons and considered the results of Haas as due to competitive reactions between various proteins among themselves and for the acid substrate. The reaction involved in the development of the turbidometric method by Kass and Seastone (1944) are somewhat confirmatory of Meyer's suggestion. In a recent note, Goldberg and Haas (1947) reported that his anti-hyaluronidase is a serum protein consisting of heat labile and thermostable components; neither of them is active singly.

**b. Neutralization of Hyaluronidase or the Permeability Factor by Specific Immune Serum.** Most of this work was performed by Mc-



Clean, and McClean and Hale. McClean (1936) found that the activity of the permeability factor of the *welchii* filtrate was not inhibited by an amount of antitoxin sufficient to neutralize the specific toxin action. It was noticed that the addition of the antitoxin caused a diminution in the immediate diffusion of the intracutaneous bleb, though the increased spread of toxins or ink used as indicator was never completely inhibited. Normal horse serum in the same concentration as the antitoxic serum did not prevent immediate diffusion, and it was concluded therefore that the inhibition might be due to an antibody. This apparent incomplete inhibition might have been due to the toxic effect of the toxin present in the filtrate, as was observed by Chain and Duthie (1940). In contrast, the immune sera prepared by McClean (1936) in rabbits by subcutaneous injections of *welchii* toxoid followed by toxin during a period of two months almost completely inhibited the activity of the diffusing factor. One volume of a 1:10 dilution of the antitoxic serum caused inhibition of the spread of a five times minimal diffusing solution. All four immune rabbits were found to suppress the immediate diffusion of an intracutaneous bleb in their skins, showing that animals possessed immunity against the diffusing factor. Anti-diffusing rabbit serum against *welchii* toxoid exercised no inhibitory action on the testicular extracts, which showed a high degree of specificity.

McClean and Hale (1941) likewise found that antihyaluronidase serum neutralized the enzymic activity. In the observation on the liberation of N-acetylglucosamine, the enzyme from *vibrion septique* was the only one used. In observations on the viscosity-reducing activity, both *Cl. welchii* and *vibrion septique* enzymes were set up with homologous and heterologous antiserum and normal serum, using a substrate of mucoprotein derived from umbilical cord. Equal volumes of undiluted serum and the enzyme preparations were mixed, and left at room temperature for 30 minutes before addition to the substrate. The amount of acetylglucosamine liberated was estimated after an 18 hour incubation at 37°C. In both experiments, the inhibition of enzymic activity was complete and strictly specific; the heterologous antisera and normal serum exerted no effect.

The diffusing activity of these enzymes in the skin occurred at a much higher dilution than that at which any *in vitro* viscosity-reducing activity could be demonstrated. These antisera only completely in-

hibited a limited number of minimal diffusing doses of diffusing factor in the skin and this probably explains the failure of Meyer, *et al.* (1940) to demonstrate inhibition of diffusion with pneumococcal antiserum.

Duran-Reynals (1939) as well as Favilli (1940) reported that antivenomous serum inhibited or inactivated both the toxic and the spreading factors of venom. Favilli found that the mucolytic activity of the venom enzyme is also completely suppressed when specific antiserum in an appropriate amount is added to the solution of venom; the addition of normal horse serum, on the contrary, had no inhibitory action. McClean (1943) found that sera which inhibit the diffusing and viscosity reducing activity of these enzymes also inhibit mucin clot prevention. Sera prepared against enzymes obtained from *Cl. welchii* and *Vibrio septique* are species- but not type-specific. Those obtained against streptococcal enzymes are group- but not type-specific. A serum prepared against diffusing factor from bull's testis inhibited this enzyme, but did not inhibit testicular enzyme from the mouse, or any of the bacterial enzymes.

Leanard and Kurzrok (1945) reported the results of a study on the effect of normal and antihyaluronidase immune serum on the dispersion of follicle cells by hyaluronidase. Normal rat serum from either sex prevented this effect by the enzyme. One to 10 dilution of normal serum inhibited the reaction only slightly. The antisera prepared against bull testes extracts containing hyaluronidase inhibited in much higher dilution the dispersion of the follicle cells of the rat *in vitro* by hyaluronidase. On the other hand, ova from these immunized rats were affected by hyaluronidase at the same rate as ova from normal rats.

## E. ANTIBODY AGAINST PROTEOLYTIC ENZYMES

### 1. Antibody Against a "Trypsin" Preparation\*

Achalme (1901) reported that a trypsin preparation produced pathological effects in guinea pigs. He believed that these effects were associated with the proteolytic property of the enzyme preparations. Im-

\*There is a long list of controversial studies on this subject. In this respect the following recent study is of interest. Our comment on this study applies to previous studies as well.

In a series of three articles on the "Antiproteolytic Activity of Serum," Grob (1943)

munization of guinea pigs with purified trypsin preparations produced sera which inhibited the tryptic activity of the preparations *in vitro* and protected guinea pigs against their toxic effects.

The purification of trypsin was carried out as follows: A five per cent suspension of commercial powdered swine pancreatin was incubated at 37°C. for 24 hours in the presence of chloroform to prevent contamination and fermentation reactions. It was then filtered under sterile

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reported on the properties of the sera of rabbits which were daily treated with crude trypsin via intramuscular, intravenous, subcutaneous and oral routes. He followed the rise and fall of the antiproteolytic activity of these sera over a period of several weeks.

Grob (Article I) believes that the possibility that antiproteolytic activities of the above sera are due to antitrypsin antibody is unlikely. He stated that outside of a weak precipitin reaction with serum of animals that had received trypsin intravenously, they were all negative; and the positive precipitin reactions showed no parallelism to antiprotease activity. According to him, the weak precipitin reactions may have been due to impurities in crude trypsin used for injections. He refers to the observations of Ten Broeck (1934, see also Part I of this treatise) who failed to obtain precipitin reactions with sera prepared against crystalline trypsin. Ten Broeck reported, however, having obtained positive Dale anaphylactic tests in guinea pigs sensitized with five times crystallized trypsin, chymotrypsin and chymotrypsinogen. Ten Broeck stated also having shown a differentiation in this manner between the enzymes as well as between them and their respective precursors. These facts show that the actively immunized guinea pigs contained specific antibodies against these crystalline enzymes.

The failure to obtain a strong precipitin reaction may possibly be explained in the following manner. It is a well-known fact that a small molecular weight hapten inhibits the precipitin reaction between an antibody and the conjugated antigen of which the hapten is a component. (For non-specific inhibitors of precipitin reactions see also Goebel and Hotchkiss, 1937.) It would therefore appear possible that the normal serum inhibitor (polypeptide) combines with trypsin, forming a compound (see Kunitz and Northrop, 1936; Schmitz, 1938) which prevents the union between trypsin and its homologous antibody. In such studies it would be necessary to separate the inhibitor from the globulin fraction before performing the precipitin reaction. Such a critical test is absent in the study by Grob.

This study lacks also the following critical tests. An electrophoretic separation of sera into the albumin fraction (containing inhibitor polypeptide) and the globulin fraction (containing antitrypsin antibody), as performed by Smith and Lindsley (1939), would have enabled Grob to determine the relative quantities of these two substances. Or at least an attempt could have been made to separate them by some means of fractionation. Another factor to be considered is the species specific relationship of the trypsin used as antigen and the animals used for immunization (see Part V). A study dealing with controversial questions necessitates likewise the use of highly purified antigens.

To evaluate the results and interpretation of Grob, it might be of interest to refer the reader also to the observations of Maschmann (p. 256), Pozerski and Guelin, and Smith and Lindsley (p. 261) who differentiated the trypsin inhibitor from the specific antiproteolytic antibodies. Grob does not make reference to these workers.

Further to interpret Grob's results (Article III) in an accurate manner, the reader is referred to that section of this treatise which deals with the subject of "The Resistance of the 'Living' Protein Molecule to Proteolytic Enzymes," and the ready hydrolysis of denatured proteins and the proteins of mechanically injured cells by proteolytic enzymes.

conditions at 35° to 38°C. through a regenerated porous porcelain filter. The preparation thus obtained had the following properties: It dissolved egg albumin and fibrin, digested milk, liquefied gelatin, hydrolyzed starch, (apparently contained  $\alpha$ -amylase), and monobutyrin, (apparently contained lipases).

*Immunization of Guinea Pigs.* Adult guinea pigs were given 5 ml. of enzyme extract intraperitoneally in small doses. During the next two days they received 10 ml. each day in small doses. On the 4th day they were given 2 ml. extract, and the injections were continued every other day by increasing the volume by 2 ml. each time. On the 10th day 10 ml. more of the extract was given. As stated by Achalme the animal thus received twice the fatal dose.

a. **Antitryptic Property of Guinea Pig Serum Immunized with "Trypsin."** The enzyme preparation at a certain concentration exercised a clotting effect on milk which might have been due to the presence of pepsin or chymotrypsin or both (Northrop 1939). The inhibitory action of immune sera on the milk clotting phenomenon by the enzyme preparation was studied. The measurement of the clotting of the milk was possible if the proteolytic activity of trypsin, which immediately dissolved the clots formed, was inhibited by antitrypsin immune serum. Preliminary experiments consisted of determining the potency of the serum under various conditions. Achalme stated he had convinced himself that normal or immune guinea pig serum, collected under sterile conditions as soon as possible by separating from the clotted blood, did not affect the milk. Fifteen drops of these sera added to 5 ml. of milk after standing for 48 hours at 45°C. produced neither clotting nor digestion: pancreatin alone did not clot the milk. At the most it produced a fine precipitate of casein which dissolved immediately. In contrast, the addition of immune serum produced a real massive clot followed by its dissolution. However, in the presence of titrated amounts of trypsin and antitrypsin serum rapid clotting took place with the retardation of incomplete dissolution of the clot after a prolonged period.

The serum of a normal animal possessed only a weak antitryptic activity in the above experiments. Achalme stated that even one volume of normal serum is insufficient to neutralize the effect of one volume of trypsin. In contrast one volume of immune serum neutralized the activity of up to 8 volumes of trypsin solution.



TABLE XI

Drops of immune serum added	Drops of trypsin added	Results of effect on 3 ml. of milk
1	10	The digestion was faster than in the control tube.
1	6	The digestion was the same as in control tube.
1	4	Rapid clotting but the dissolution of clot proceeded at a slower rate.
1	2	Slow clotting, incomplete dissolution, unchanged after 48 hours.
1	1	No change.

Control=6 drops of trypsin solution digested 3 ml. of milk at 45°C. during a 60-minute period.

**b. The Toxic and Lethal Action of the Trypsin Preparation.** Subcutaneous injection of 3 to 4 ml. of the preparation produced, at the injected area, an inflammatory lesion; within 15 to 20 hours a characteristic slough was observed. A larger dose produced death within 36 hours. Histological study of the necrotic lesion at different phases of formation revealed paralysis of the blood capillaries, which dilated and permitted the passage of large volumes of serum and red cells.

Due to the complex nature of the extract, Achalme found it difficult to isolate the active principle responsible for the toxic effects. However, he was convinced that the effects were due to trypsin. Amylases and lipases were stated not to exercise pathological effect. A filtered extract of papain, on the other hand, produced toxic effects identical with that produced by trypsin, which provided him with confirmatory evidence that the proteolytic property of the preparation was responsible for the pathological effects. He corroborated these findings with results obtained with bacteria which exercised high degrees of proteolytic activity.\*

The above cited results obtained by Achalme appear to be corroborated by the findings of other investigators. Ten Broeck (1934) found

\*Pozerski and Guelin (1938b) studied thirteen different bacteria with varying degrees of proteolytic activity with respect to their slough producing capacity. In contrast to Achalme's findings they could not establish any direct correlation between these two properties of bacterial proteases.



that injections of chymotrypsinogen into female guinea pigs produced no visible effect, but animals receiving trypsin and chymotrypsin showed necrotic areas at the site of inoculation, and several of them died. Chymotrypsin seemed to be more toxic than trypsin. Eagle and Harris (1937) reported that trypsin injected intravenously into rabbits caused almost immediate death. Large blood clots were found in the heart and large veins. The free fluid was non-coagulable, and contained no demonstrable fibrinogen. They stated that trypsin thus had initiated blood coagulation *in vivo* as readily as it had done *in vitro*.

In another experiment 10 ml. of a 5 per cent solution of crude trypsin were injected intravenously into a rabbit weighing 2 kg. The animal died in convulsions in two minutes, and large clots were found in the great veins two minutes after death. Likewise 1 ml. portions of a 5 per cent solution of crude trypsin were injected into a rabbit at 10-minute intervals. One and one-half minutes after the fourth injection convulsions began and death followed in one minute. An autopsy six minutes later revealed that the right ventricle and both auricles were filled with solid clots.

**c. Neutralization of the Toxic Effects of Trypsin with Immune Serum.** A mixture of 15 drops of immune blood and 4 ml. of trypsin solution injected by Achalme into small guinea pigs was non-toxic. There was no local effect, no vasomotor paralysis and no swelling of the spleen. The injected mixture was immediately adsorbed. These facts were believed by Achalme to have shown the complete neutralization of the toxicity of trypsin by its homologous antibody. A neutralizing effect on the activity of papain by anti-trypsin immune sera in *in vitro* and *in vivo* experiments was not observed.

**d. Antibody Against Papain.** Several controversial works reviewed by Walton and Segura (1932) have been published. A recent study by Haas (1940) shows conclusively the formation of a specific antibody capable of inhibiting the proteolytic activity of papain preparations. The enzyme preparation with which Haas experimented was obtained by subjecting *papain puriss* (Witte) to repeated fractional acetone precipitation. It readily dissolved in water. For immunization and serological, etc. experiments the solutions were filtered through EK-Seitz filters.

Guinea pigs immunized with a total of 2 to 3 mg. papain during a period of 21 days experienced fatal anaphylactic shock with 0.2 to

1 mg. papain injected intravenously. Normal guinea pigs showed no anaphylactic symptoms on receiving 5 to 10 mg. papain preparation. Using the Schultz-Dale technique the uterus of sensitized guinea pigs contracted strongly in the presence of 1 mg. papain. In precipitation tests antipapain rabbit sera reacted with papain but not with pepsin. Antipepsin sera reacted weakly with pepsin but showed no reaction with papain. In complement fixation experiments antipapain serum, in 1:20,000 dilution, inhibited the hemolysis of red cells.

In view of the fact that papain used by Haas was perhaps not a single entity, or may have been contaminated with other protein impurities, the above results may not be taken as conclusive evidence that papain itself was responsible for these reactions. The following experiments which deal with the inhibition of the proteolytic activity of the papain preparations with specific immune rabbit sera show conclusively, however, that a specific antibody against papain was produced in the blood of rabbits immunized with these preparations.

*Inhibition of the Proteolytic Activity of Papain with Antipapain Sera.* The maximum antigen-antibody reaction indicated by the amount of precipitate produced was found to cause the highest degree of inhibition of the proteolytic activity.

Four ml. of inactivated serum (immune or normal serum) treated with 1 ml. of 2 per cent papain solution were incubated at 40° for one hour. The mixture, after treating with 1 ml. of 0.2 M disodium citrate solution and 0.4 ml. of 0.5 per cent potassium cyanide solution (neutralized with 0.16 N HCl solution against methyl red) was made up to a volume of 20 ml. with distilled water. To this was added 4 ml. of 5 per cent casein or 4 ml. of 3 per cent gelatin solution of pH 4.8 to 4.9. The reaction mixture was incubated at 37°C. for 44 hours. At the end of 20 and 44 hour periods sterile samples were taken and titrated with alcoholic 0.01 N sodium hydroxide solution to determine the increase in acidity as a result of proteolysis.

In this series of experiments papain-antipapain precipitate was left in the reaction mixture. In another series of experiments the precipitates resulting from the papain-antipapain reaction were centrifuged and 5 ml. of the clear supernatant solution was tested for proteolytic activity as described above. In all these experiments reaction mixtures were held under sterile conditions. The results showed that, in contrast to the results obtained in the presence of normal rabbit sera, the

hydrolysis of casein or gelatin was completely absent in the reaction mixtures containing the substrate and the supernatant which was obtained by centrifuging off the papain-antipapain precipitates. In the reaction mixtures containing the papain-antipapain precipitates the inhibition of the hydrolysis of casein at the end of 18 and 42 hour periods was, respectively, 72.5 and 58 per cent; similarly the inhibition of the hydrolysis of gelatin at the end of 20 and 24 hour periods was, respectively, 71.5 and 72.6 per cent. In view of the difference in the degree of inhibition of the hydrolysis of casein or gelatin arising from the difference in the details of the two methods employed, it would appear that when the papain-antipapain precipitate is left in the reaction mixture a certain degree of dissociation may have taken place. Under these conditions the substrates may have competed with the antibody for the active group (or the combining site) of the antigen causing partial displacement of antibody and thus reducing the degree of inhibition from 100 to ca. 70 per cent. An alternative explanation, of course, would be that combination of papain with antibody blocks the enzymatically active groupings only up to about 70 per cent of maximal activity.

**e. Antibody Against the Proteolytic Activity of Snake Venom.**

Githens (1941) reported the result of a study of the proteolytic activities of snake venoms from 26 species. Using gelatin as substrate he found that there was no significant difference in the speed of digestion by different venoms, and the comparison of different lots of venoms of the same species showed close agreement, but he observed that venoms of different species of pit viper show great differences. In studies on the antiproteolytic property of antivenins Githens found that 0.3 ml. of antivenin prevented the proteolytic effect of as much as 1 mg. of some snake venoms, and was entirely ineffective and irregular with others. Both crotalidic and cascabel antivenins showed stronger action against the venoms of a true viper, the daboia, and of an elopine snake, and the Australian tiger snake, than against those of many pit vipers. Eagle (1937, 1939) studied the proteolytic activity of venoms using gelatin as substrate. Comparing plasma clotting and gelatin digesting activities of certain venoms, he found that the venoms arranged in order of their gelatin-splitting activity showed also proportional clotting activity. When the proteolytic activity of certain venoms was found to be below a certain level they also failed to mani-

fest any effect on fibrinogen. On the other hand, some of the venoms which manifested a high degree of proteolytic activity rendered the fibrinogen non-clotting even by thrombin. These facts were interpreted by Eagle as substantiating the hypothesis that the clotting, like the digestion, is caused by one of the proteolytic enzymes in the venom (see, however, the section on the mechanism of fibrin clot formation, p. 278).

**f. Proteolytic Activity of the Toxin of *Cl. Welchii*.** As stated above, Maschmann found that the toxin fraction of the culture filtrate of *Cl. welchii* hydrolyzed gelatin and was not inhibited by the "trypsin-inhibitor" present in normal sera. Taking advantage of this fact he demonstrated that antitoxic horse serum strongly inhibited the proteolytic activity of a toxin preparation. Since he did not claim that the toxin he experimented with was a homogeneous substance, the question as to whether the proteolytic activity of the preparation is a property of the toxin molecule or not must be left open for the present.

Since  $\beta$ ,  $\delta$ ,  $\theta$  and  $\epsilon$ -toxins were found by MacFarlane, *et al.* to produce no opalescence in human serum or lecitho-vitellin, the proteolytic activity of the toxin preparation appeared to be associated with the  $\alpha$ -toxin. The toxin was a dry preparation prepared from dialyzed culture

TABLE XII

Reaction systems	Hydrolysis of gelatin (ml. of N NaOH)	
	No cysteine	Cysteine
1 ml. of dry toxin solution (2.7 mg.) + gelatin solution	0.81	0.75
1 ml. of dry toxin solution (2.7 mg.) + 0.0016 ml. immune serum + gelatin solution (The mixture not fatal to, but made the test animal sick)	0.72	0.74
1 ml. of dry toxin solution (2.7 mg.) + 0.016 ml. of immune serum + gelatin solution (The toxin was overneutralized)	0.40	0.47
1 ml. of dry toxin solution (2.7 mg.) + 0.016 ml. of normal serum + gelatin solution	0.87	0.98
Per cent inhibition*		52.0

\*Per cent difference between the activity of overneutralized toxin and that in normal serum.



fluid. The optimum activity was at pH 7. Normal horse serum in amounts of 0.4 to 2 ml. (1 to 10 dilution) had no effect on the enzyme activity.

The absence of inhibition of the proteolytic activity of the toxin by normal horse serum and the inhibition of its activity by antitoxin was determined (Table XII).

The volume of the reaction systems was made to 10 ml. (0.40 g. of gelatin); pH=7 (without buffer);  $t=40^{\circ}$ ; period 24 hours; toluol and cysteine were added under an atmosphere of nitrogen. The test solution for titration was 2 ml.

The above data show that the proteolytic activity of the toxin preparation of *Cl. welchii* was inhibited 52 per cent.

## 2. Antibody Against Bacterial Proteinases

Von Dungern (1898) reported that the sera of persons infected with anthrax bacillus, cholera vibrio and staphylococcus contain antiproteolytic antibody. These antisera inhibited the hydrolysis of gelatin by the proteolytic enzymes of these microorganisms. The enzyme preparations used in these tests were either broth culture filtrates or bacterial extracts which had been precipitated with alcohol. In control tests, gelatin was hydrolyzed by these preparations. In contrast, in the presence of 0.5 to 0.002 ml. of specific immune sera, the hydrolysis of gelatin by the enzyme preparations was completely or partially inhibited. Also, the sera of animals immunized with the enzyme preparations strongly inhibited their activity. Normal serum exercised no inhibition. The sera of two patients suffering seriously from osteomyelitis inhibited twenty times as much staphylococcal proteolytic enzyme as was inhibited by normal human serum. The inhibitory action of the antisera was specific.

Dochez and Avery (1916) reported that antipneumococcal serum exercised marked inhibitory action on the growth of homologous pneumococcus during a three hour growth period as compared with the absence of inhibitory effect with normal horse serum. The inhibition by homologous immune serum was greater than by heterologous (species) immune serum. These facts showed that, as they concluded, some property of immune serum adversely affected the circumstances of multiplication.



In experiments to determine what bacterial enzymes are inhibited by antipneumococcal antisera, they found that the antisera diminished in some instances almost to the point of extinction, the production of amino-acids by the organism. These findings showed that the proteolytic enzymes of pneumococcus were inhibited by the antisera.

a. **Immunity Against Proteases of Various Bacteria.** In a work similar to that of Dochez and Avery, Wohlfeil (1936) studied the proteolytic action of anthrax bacillus, *B. subtilis*, cholera vibrio, diphtheria bacillus, staphylococcus and *B. proteus* on serum proteins. As a result of their activities, the residual or non-precipitable (with trichloroacetic acid) nitrogen increased. His calculations showed that one billion staphylococci under optimal conditions were capable of hydrolyzing 3.79 g. of protein to peptones and amino acids in twenty-four hours. The same number of proteus bacilli were able to hydrolyze about 4.86 g. of blood serum proteins. On the basis of these observations, he concluded that the increase of residual nitrogen in the sera of infected patients is due to the proteolytic activity of bacteria. He found that immune sera inhibited bacterial autolysis. As a result of this, the action of bacterial cell proteinases on serum proteins was inhibited, as shown by the decrease of residual nitrogen in a system containing bacteria and immune serum. The amount of residual nitrogen in the presence of immune serum was considerably less than in normal serum. Immune anti-enzymes against cell proteinases were present in antisera against staphylococci and *B. proteus*. The inhibitory action of these sera was specific. Immunity against the proteolytic activity of the culture fluids of *B. subtilis* and *B. pyocyaneus* was also reported by Bertiau (1914). These filtrates were concentrated *in vacuo* at low temperature and repeatedly precipitated with alcohol. The final products were dissolved in saline and filtered. The minimum active amount of the enzyme solution from *B. subtilis* was 0.1 ml., determined by incubating with the substrate for one hour at 37°C. That of *B. pyocyaneus* was 0.05 ml. Incubation longer than one hour made very little difference. Bacteria grown in broth for two days yielded the maximum amount of enzyme. The enzyme of *B. pyocyaneus* was stable at 60°C. and that of *subtilis* began to lose its activity even at 56°C.

In a systematic study in which the conditions of experimentation were critically controlled, Bertiau found that the activity of enzyme preparations from these bacteria was inhibited by antienzyme immune

sera. Rabbits immunized subcutaneously or intravenously with filtered culture fluid containing carbolic acid yielded sera of equal potency. The rabbits were given injections every four to five days for two months, and in some cases, for three months.

TABLE XIII

*Inhibitory Action of Antisera on the Hydrolysis of Gelatin by Enzyme Preparations*

Pyocyaneus Immune Serum+0.5 ml. of Enzyme Solution										
Systems	Ml. of serum used	0.8	0.4	0.2	0.1	0.05	0.025	0.012	0.0062	0
1	Immune serum unheated +enzyme,	— —	— —	— —	— —	— —	— —	±	++	++
2	Immune serum heated at 100° +enzyme,	++	++	++	++	++	++	++	++	++
3	Normal rabbit serum A +enzyme,	++	++	++	++	++	++	++	++	++
4	Normal rabbit serum B +enzyme,									

— — sign indicates absence of hydrolysis.

++ sign indicates hydrolysis.

The results show that 0.025 ml. of immune serum completely inhibited the proteolytic activity of 0.5 ml. of the enzyme solution. In contrast, normal sera showed no inhibitory action.

The anti-enzyme (from *B. subtilis*) immune serum behaved similarly, though somewhat less strongly. The antisera had no inhibitory action on trypsin or other bacterial proteinases. *B. subtilis* anti-serum had no action on *B. pyocyaneus* and *vice versa*.

**b. Antibody Against Streptococcal Proteinase.** According to Elliott (1945) Group A *Streptococcus pyogenes* under certain conditions produce an extracellular proteinase which digests the type-specific M

antigens of streptococcal strains of Group A. It digests also fibrin, casein, milk, gelatin and streptococcal fibrinolytic factor. The enzyme is activated under the reducing conditions of active bacterial growth and by cysteine, glutathione, thioglycollic acid, and potassium cyanide; it is inactivated by iodoacetic acid. This enzyme was differentiated from streptococcal hemolysin, hyaluronidase or fibrinolytic factor.

Todd (1947) studied the serological specificity of the streptococcal proteinase. He found that the antiproteinase activity of serum from horses immunized with streptococcal proteinase was increased as much as 200 times above the original level. The antiproteinase sera were ineffective against other proteolytic enzymes. That the increased antiproteinase activity of the immunized animals was due to the presence of a specific antibody was shown by a lack of a similar increase in antiproteinase activity in animals immunized with tetanus toxoid, diphtheria toxoid and erythrogenic streptococcal toxin. The sera of animals immunized with these toxoids contained respectively 10, 10, and 0 units of antiproteinase per ml., while a sample of serum from a normal horse in the same stables contained 10 units of antiproteinase per ml. A horse was immunized for 15 months with streptococcal erythrogenic toxin. Both before and after immunization, the serum of this horse contained no demonstrable antiproteinase. A sample of concentrated serum from the pooled bleedings of horses immunized with erythrogenic toxin contained 3,300 units of antibody to toxin and only 20 units of antiproteinase. These experiments show that antiproteinase activity does not develop in the sera of horses immunized with antigens which do not contain streptococcal proteinase.

Antiproteinase is in the globulin fraction of both normal and immune sera. Heat abolishes the normal trypsin inhibiting activity of serum but was found to have no effect on the antiproteinase activity of the serum. The trypsin inhibitor had no demonstrable neutralizing activity for streptococcal proteinase.

c. Differences of the Proteinases of *Cl. Sporogenes* and *Cl. Histolyticum* and the Specificity of the Anti-Proteinases. Blanc and Pozerski (1920) studied the proteolytic enzymes of *Cl. sporogenes* and *Cl. histolyticum* in comparison with pepsin, trypsin and papain. The bacterial proteinases, unlike pepsin, were inactive at pH 5.5. They hydrolyzed protein to the amino acid stage whereas pepsin stops at the peptone stage of protein hydrolysis. The bacterial proteinases, like

papain, digested raw muscle, but were inactive against raw egg albumin and serum; in this respect, they behaved like trypsin. Like trypsin, they were active in neutral or weakly alkaline medium. Coagulated albumins were hydrolyzed to amino acids. Like trypsin, the bacterial proteinases were precipitated from their solutions by 0.8 per cent safranin.

The hydrolysis of gelatin by *Cl. sporogenes* proteinase was inhibited by 0.1 ml. of normal horse serum, which was evidently due to the trypsin inhibitor normally present in the serum. In contrast, as much as 1 ml. of normal horse serum had no inhibitory action on the proteinase activity of *Cl. histolyticum*. This highly active digestive property of the latter bacterium was assumed to be responsible for the tissue damage caused in an infected host when a culture of this bacillus is injected into the muscle of a guinea pig.

The gelatinase activity of *Cl. sporogenes* was inhibited in the presence of raw egg white but that of *Cl. histolyticum* was not at all affected (Pozerski and Guelin, 1938a).

Horses immunized (M. Weinberg) with the filtrates of *Cl. histolyticum* and *Cl. sporogenes* yielded specific antisera. The proteinase activity of *Cl. sporogenes* was not inhibited at all by the antiproteinase immune serum against *Cl. histolyticum* and *vice versa*.

Pozerski and Guelin (1938b) also studied the proteolytic activity of thirteen anaerobic bacilli from M. Weinberg's collection at the Pasteur Institute, Paris. Of these, only three failed to show any activity; the other ten anaerobes manifested proteolytic activity of varying intensity. Proteolytic preparations from *Cl. histolyticum*, *vibrio septique* and *Cl. perfringens (welchii)* D, B and C manifested escharotic properties when injected subcutaneously into guinea pigs. A similar property with the proteolytic filtrates of the other bacilli was not obtained.

Smith and Lindsley (1939) studied the proteolytic activity of bacteria of the gas-gangrene group which cause much tissue damage. They compared the proteolytic activity of pathogenic and non-pathogenic members of this group. In this study, similar in many respects to that by Blanc and Pozerski (1920) and Maschmann (1937), they found that normal serum inhibited a proteolytic enzyme found in non-pathogens, but the activity of the pathogens was not inhibited. Of the pathogens tested, *Cl. histolyticum*, *Cl. welchii*, and *Cl. oede-*

TABLE XIV

d. The Ability and Inability of Normal Serum "Trypsin-Inhibitor" and Raw Egg White to Inhibit the Proteinases of Pathogenic and Non-Pathogenic Bacteria.

Bacterial strains	Inhibition by:		Pathogenicity
	Normal serum	Raw egg white	
<i>Cl. histolyticum</i> , smooth	0	0	Appears in gangrenous process of man; guinea pig, rabbit and mouse are susceptible.
<i>Cl. histolyticum</i> , rough	+ +	— —	Non-pathogenic.
<i>Cl. sporogenes</i>	+ +	+ +	Non-pathogenic, no exotoxin produced.
<i>Cl. aerofetidum</i>	+ +	— —	Non-pathogenic.
<i>Cl. botulinum</i> , types A & B	+ +	— —	Does not multiply in the body, acts entirely by its powerful toxin produced <i>in vitro</i> .
<i>Cl. fallax</i>	+ +	— —	Pathogenic for guinea pig, mouse when freshly isolated, but pathogenicity rapidly disappears even in dry form.
<i>Cl. oedematis-maligni</i>	0	— —	Pathogenic: guinea pig, mice, rabbits; causes gas gangrene in man; potent toxin producer.
<i>Cl. putrificum</i>	+ +	— —	Non-pathogenic.
<i>Cl. bifementans</i>	— —	+ +	Non-pathogenic.
<i>Cl. welchii</i>	0	— —	Chief agent in causing gas gangrene in man and animals.
<i>Cl. sordelli</i>	0	±*	Pathogenic: found in gas gangrene of man and sheep; rabbit, guinea pig and mouse susceptible.
<i>B. pyocyaneus</i>	+ +	— —	Low pathogenicity in man; subcutaneous injection gives rise to fever and local abscess in rabbits.
<i>B. prodigiosus</i>	+ +	— —	Non-pathogenic.
<i>B. mesentericus</i>	+ +	— —	Non-pathogenic.
Trypsin	+ +	Action delayed	Kills rabbits injected intravenously.

+ + = inhibition      0 = no inhibition      — — = data lacking.

\*Pozerski and Guelin (1938a) reported that raw egg white inhibited the hydrolysis of gelatin by the proteinase of this organism. However, coagulated serum, which contains the thermostable "trypsin-inhibitor," is hydrolyzed by this organism.



*matris-maligni* were capable of hydrolyzing proteins in the presence of normal serum. These facts suggested to them that this characteristic is an important one in pathogenesis, enabling the members of this group to establish a foothold in the body of the host, and to obtain those breakdown products of protein which are their main source of energy. In comparing the proteolytic activities of rough and pathogenic *Cl. histolyticum* strains, they found that the rough strain, as well as trypsin, were inhibited by normal serum; in contrast, the pathogenic strain was not inhibited.

Immune sera prepared by the injection of filtrates of smooth *Cl. histolyticum* into rabbits inhibited the enzymes of this organism. This inhibition was specific. The anti-proteinase immune serum to this strain did not inhibit the proteolytic activity of *Cl. sporogenes*, *Cl. welchii*, or even the rough strains of *Cl. histolyticum*. This experiment was performed with antiserum which had stood sufficiently long in the cold to have lost its ability to inhibit the proteinases of the non-pathogenic organisms. While the trypsin-inhibitor was shown to be found in the albumin fraction by electrophoretic fractionation, the immune body against bacterial proteinase was found in the globulin fraction.

The question as to whether there is a causal relationship between the ability of certain bacteria to resist the inhibition of normal serum and pathogenesis cannot as yet be stated with certainty. However, the results of the studies of the above mentioned several investigators are presented in Table XIV (p. 262) to enable the reader to evaluate for himself the available data.

## F. FIBRINOLYSIS

### 1. Serum Proteinase as Fibrinolytic Enzyme and the Role of Bacterial Fibrinolytic Factor

**a. Comment on Nomenclature.** The dissolution or digestion of a plasma clot or fibrin with or without the participation of bacterial factors is known as *fibrinolysis*. The bacterial factors which participate in *fibrinolysis* have been known as *fibrinolysins*. It has now been proposed that new terminologies be adapted.

The enzyme which is found in serum and brings about the lysis of fi-

brin or serum clot has generally been known as *serum tryptase* or *serum protease*. Since serum proteolytic enzyme has been found, in some respects, to differ from the usual trypsin, the use of the term serum protease would seem to merit more serious consideration. Christensen (1945, 1946) and Christensen and MacLeod (1945) have proposed the name *plasmin*, and Loomis, George and Ryder (1947) *fibrinolysin*. The former term appears to account for the properties and the origin of the serum enzyme, the latter term is too confined in its scope for the reason that serum enzyme is capable of digesting also gelatin, casein, hemoglobin and fibrinogen. The normal inactive state of serum protease has been called *plasminogen* (Christensen, 1946), and *pro-fibrinolysin* (Loomis, *et al.*, 1947). These names convey, according to our present knowledge and understanding, erroneous meaning. The chemical reactions involved in the conversion of the inactive state of serum protease into the active state, and our critical information concerning the chemical structural and origin of the inactive form of this enzyme exist, as yet, in a state inadequate to justify the adoption of these terms here. Christensen (1945) attributes to the bacterial factors (fibrinolysins) a *kinase* role in fibrinolysis, and Loomis *et al.* (1947) have named it *streptokinase*. For experimental reasons as discussed below, the introduction of these terms would seem to be premature. The term streptokinase is particularly unfortunate for the same reason and also for the reason that many other species of bacteria have been reported to elaborate the same or similar factors.

It would, therefore, seem desirable that, at present, the adoption of such terms be deferred until our understanding of the basic reactions and the specific enzymes involved in blood clotting and lysis of the products are more specifically characterized. We therefore find it expedient to use here the term *fibrinolysis* for the process which involves the digestion of blood clot or fibrin; *serum protease* for the enzyme which catalyzes fibrinolysis, since it is a proteolytic process; and the substances which are derived from various bacteria capable of contributing to fibrinolysis as *bacterial fibrinolytic factors*, or, merely, the term *bacterial factors* to be used solely in conjunction with fibrinolysis.

**b. Digestion Products of Fibrin and Fibrinogen.** Information is lacking concerning the specific question of whether the lysis of fibrin occurs because of the opening of the primary bonds (see section on Thrombin) which were formed during the conversion of fibrinogen to

fibrin, or whether the digestion of the intact portions of the fibrin-forming fibrinogen units results in the dissolution of fibrin structure. Working on the lysis of fibrin clots by the action of streptococcal fibrinolytic factor, Garner and Tillett (1934b) reported that they were unable to detect significant evidence of proteolysis during fibrinolysis. They also reported that if fibrinogen is incubated with bacterial fibrinolytic factor for a brief period, the fibrinogen can no longer be converted to fibrin upon the addition of thrombin. Apparently, the serum lytic factor (protease) as contaminant in fibrinogen having been activated by bacterial factor had produced a deep-seated change in the fibrinogen molecule.

Seegers, Niefert and Vandenbelt (1945), working with a fibrinogen preparation yielding 96 per cent fibrin, found that when the fibrinogen solution was allowed to stand at room temperature for 6 days it completely lost its fibrin-forming property on treatment with thrombin. Apparently, a trace of inactive serum protease present as contaminant was activated by treatment with alcohol, or chloroform, etc. used during the preparation of fibrinogen. The fibrin prepared from whole fibrinogen by the action of thrombin was likewise allowed to stand at room temperature until virtually all the fibrin had gone into solution. The decomposed solutions of fibrinogen and fibrin were dried from the frozen state and then analyzed electrophoretically, and fractionated chemically into  $\alpha$ - and  $\beta$ -components. Both fibrinogen and fibrin decomposition products yielded these two derivatives.  $\beta$ -Fibrinogen derivative, one of the two components, showed electrophoretic properties similar to that of whole fibrinogen. It was heat coagulable at 51°C., precipitable with ammonium sulfate and had an isoelectric point near pH 5.5, which is the isoelectric point also of fibrinogen.  $\beta$ -Derivative of fibrinogen, present in smaller concentration, was non-coagulable with heat, soluble in ammonium sulfate, and its isoelectric point was pH 4.2. Despite the similarities between the  $\alpha$ -derivative and whole fibrinogen the former lacked the clot forming property, indicating that degradation of both the fibrinogen molecule and the fibrin structure had taken place. No data were submitted concerning the other possible digestion products of smaller molecular weight.

Christensen (1945) studied the extent of the digestion of fibrin clot and fibrinogen by measuring: (a) the decrease in acid-precipitable nitrogen; (b) the increase in acid-soluble "tyrosine"; (c) the libera-

tion of amino nitrogen; and (d) the decrease in viscosity. On the basis of careful measured results he arrived at the conclusion that fibrinolysis is incident to proteolysis of the fibrin molecule, and that proteins other than fibrin or fibrinogen can be digested.

Christensen and MacLeod (1945) reported that in the completion of the digestion of casein by serum protease there are left linkages which are split by trypsin. On the other hand, serum protease does not produce further hydrolysis of casein which has already been acted upon by trypsin, showing further differences between these proteolytic enzymes. Kaplan (1946) likewise pointed out that the streptococcal serum protease system differs in specificity from the enterokinase-trypsinogen system. Streptococcal factor did not activate trypsinogen, nor was the serum protease activated by enterokinase. Jablonowitz (1939) reported that following the action of fibrinolytic agent fibrinogen underwent alteration in its serological property. (See further Morrison (1947), and Edsall, *et al.* (1947) concerning the properties of fibrinogen.)

**c. Distribution of Bacterial Fibrinolytic Factor.** Fibrinolytic factor is reported by Tillett (1938) to be most widely elaborated by the strains of *Streptococcus hemolyticus*, particularly by the types belonging to Lancefield's Group A. It is elaborated also by Groups C and G; strains belonging to Group B, D, E, F and *Streptococcus viridans* are reported not to elaborate this factor. According to Tillett and Garner (1933) and Tillett (1935, 1938), the production of this factor is specific for hemolytic streptococci. However, the following findings show that this factor is elaborated by other streptococci and species of bacteria. Neter and Witebsky (1936) studied the effect of the concentration of carbohydrates in meat infusion broth on the production of fibrinolytic factor. They reported that the production of this factor by hemolytic streptococci may depend upon the glucose concentration, as well as the particular strain, and that there is no definite relationship between the production of the factor and the type of sugar used (glucose, mannite, salicin and lactose). They found that 31 of 40 strains of hemolytic streptococci, 35 of 47 strains of *Streptococcus viridans*, as was also shown by Tunncliffe (1936), produced the fibrinolytic factor.

All of the 31 strains of enterococcus (*Streptococcus fermenting aesculin*, a glucoside from horse chestnut) were found to produce the



fibrinolytic factor. Le Mar and Gunderson (1940), testing the activity of fibrinolytic factor of  $\beta$ -hemolytic streptococci from human and veterinary strains (swine, dog, sheep, guinea pig, rabbit, fox, duck and pigeon, bovine and horse) against their homologous fibrin and cross-testing against each of the other fibrins, reported that the group of streptococci of human origin was more actively lytic than any other, attacking all fibrins to some degree. Lysis of human fibrin by streptococci, however, was most rapid and complete of all. Likewise, each group of veterinary organisms attacked human fibrin more quickly and dissolved it more completely than it did its own homologous fibrin. Prolonged incubation (96 hours) of cultures enhanced the lytic power of most of the strains of streptococci. Complete lysis of fibrin occurred in 75 per cent of the tests in less than 30 minutes when 96 hour cultures were used as source of fibrinolytic factors.

Madison (1935) reported that of 132 strains of staphylococci, 80 per cent of all strains originally isolated from internal human lesions were capable of liquefying human fibrin. Approximately 90 per cent of all strains isolated from superficial human infections, however, and all strains from veterinary lesions were inactive by the same *in vitro* technique (see also Madison and Dart, 1936). Neter (1937) reported that staphylococci from human sources may produce fibrinolytic factor dissolving human as well as animal plasma clots. Of 43 strains of hemolytic *Staphylococcus aureus* six produced the fibrinolytic factor. On the other hand, of 10 strains of non-hemolytic staphylococci none produced the factor. Staphylococcal factor was specifically neutralized by staphylococcus antiserum. It was antigenically different from the factor elaborated by *Streptococcus hemolyticus*. Neter and Witebsky (1936) and Witebsky and Neter (1936) reported that of 78 strains of pneumococcus, 31 strains produced, while 47 strains did not produce the fibrinolytic factor. Several strains of *Escherichia coli*, *B. lactis aerogenes*, *B. friedlaenderi*, *B. pyocyaneus* and *B. proteus* were reported to produce this factor when cultured in meat-infusion broth containing 2 per cent glucose.

The observation by Neter and Witebsky (1936) that the cultures of many organisms which had been grown in a medium containing 2 per cent glucose possess anticoagulant and fibrinolytic activities, has been found by Tillett (1937) and Zinsser and Williams (1949) to be due to a pH below 5.0 resulting from the breakdown of glucose.



Zinsser and Williams (1949) tested 60 strains of Gram negative bacilli for fibrinolytic capacities. Fibrinolytic activity was found in 31 of 60 strains of *E. coli*. They found an association between fibrinolytic and hemolytic capacities of *E. coli* and virulence. Fibrinolytic capacity and virulence disappeared simultaneously in artificial media even though hemolytic capacity remained. Virulence in lactose non-fermenting groups seemed to bear no relation to hemolytic or fibrinolytic capacities. The lysis by *E. coli* was found to proceed at a slower rate than in the case of *Streptococcus pyogenes*. No cell-free extract was found to have activity. Lysis occurred only in the presence of living and metabolizing bacteria. Cysteine and adenylic acid inhibit lysis by *E. coli*, but accelerate that by streptococci.

Madison (1936) reported that 15 rodent strains of *B. pestis* are strongly active in fibrinolysis, particularly when tested with rat- or guinea pig-fibrin. He also found that one human strain of *B. pestis* yielded lytic factors of relatively high titer for ground squirrel and human fibrins. Filtrates of *Cl. histolyticum* grown on various protein-rich media were shown (Carlen, 1939) to contain highly active lytic factor. Reed, *et al.* (1941, 1943) reported that pathogenic species of gas-gangrene anaerobes produce fibrinolytic factor. Eleven species of genus *Clostridium* (some 77 strains) tested fell into two distinct groups. A majority of cultures of *Cl. welchii*, *Cl. novyi*, *Cl. septicum*, *Cl. sordelli*, *Cl. chauvoei*, *Cl. histolyticum*, *Cl. sporogenes* and *Cl. tyrosinogenes* produced fibrinolytic factor effective in lysis of fibrins from man, guinea pig, rabbit (except *Cl. sordelli*) and sheep (except *Cl. welchii*). In contrast, no cultures of *Cl. fallax*, *Cl. tertium*, *Cl. aerofoetidum* produced a measurable amount of the factor active on human, guinea pig, rabbit or sheep plasma fibrin.

According to Reed, *et al.*, in the case of human plasma, *Cl. welchii* produced the most active fibrinolysis; 83 per cent of 26 strains produced complete solution in 24 hours or less, 17 per cent produced complete solution in seven hours or less and a few in one to two hours. *Cl. histolyticum* cultures were about equally active. All the other active species required from 7 to 24 hours to complete solution of coagulated human plasma. For the most part, guinea pig and rabbit plasma were more rapidly, and sheep plasma less rapidly lysed than plasma from man by all the fibrinolytically active species of *Clostridium*. According to these investigators, the activity of the fibrinolytic factors

of clostridia resemble those of staphylococci and stand in sharp contrast to that of *Streptococcus hemolyticus*. A recent brief survey on the production of lytic factors by various bacteria is reported by Lewis, *et al.* (1949).\*

**d. Fibrinolytic Factor and Bacterial Invasiveness.** The possible role of bacterial fibrinolytic factor in the invasiveness of *Streptococcus pyogenes* has been variously considered. It has been suggested that this factor aids the lysis of clots formed in an inflamed area, thus permitting the infiltration of the invasive agent through damaged tissue into the blood stream. According to Dennis and Berberian (1934) those strains of streptococcus which elaborate fibrinolytic factor are able to invade and cause more serious conditions such as septicemia. And those strains (*Streptococcus viridans*) which are unable to elaborate this factor lack invasiveness. It may be that these explanations of the role of fibrinolytic factor represent certain of the events that take place when an infection takes the proportion of a septicemia. However, the processes of inflammation and invasiveness are too complex to permit a well-defined biochemical role, at present, to one particular factor. Our information concerning the role of various factors are as yet too meagre to define the role of each factor specifically.

**e. Antibody to Fibrinolytic Factor.** It is a known fact that the sera of patients convalescing from streptococcal infection contain specific antibodies which neutralize the function of fibrinolytic factor in fibrinolysis. Massell, *et al.* (1939) and Mote, *et al.* (1939) claimed that there are differences in the structure of fibrinolytic factors from different strains causing the formation *in vivo* of structurally different anti-fibrinolysins. Should this be true, it would mean that the fibrinolytic factors are strain specific. An extensive quantitative study of a great many streptococcal strains by Kaplan (1946) contradicts the strain specificity of fibrinolytic factors.

In a report based on a study of 404 well soldiers and 808 men admitted to the respiratory wards of the hospital, the U. S. Army Com-

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\*Of the bacterial lytic factors (fibrinolysins) those of the  $\beta$ -hemolytic streptococci are most extensively studied. The presence of lytic factors in other species of bacteria are of interest, but cannot, at present, be safely stated to be similar in action to streptococcal fibrinolysin. The question of whether or not the lysis of fibrin clots occurring with the participation of the culture filtrates of other species of bacteria is due to proteolytic enzymes, or to non-proteolytic factors cannot at present be answered. The answer must come from a study of the properties of at least partially purified preparations.

mission on Acute Respiratory Diseases (1946) published the following findings on:

- (1) The normal range of antifibrinolysin titers in healthy subjects;
- (2) The frequency with which  $\beta$ -hemolytic streptococcal infections stimulated an antifibrinolysin response; and,
- (3) The specificity of the antifibrinolysin response.

Using a standard unit of fibrinolytic factor obtained from a Group A strain of  $\beta$ -hemolytic streptococcus, a titer of 50 units or less was exhibited by 66 per cent of sera collected from normal subjects, indicating the presence of little or no antifibrinolysin. In approximately 11 per cent of the normal subjects the antifibrinolysin antibodies were considered to be elevated in that the titer was greater than 150 units. That the high antifibrinolysin titers observed in normal subjects resulted from previous experience with  $\beta$ -hemolytic streptococcus was indicated by the fact that the *antistreptolysin* titer was usually elevated in those sera which also showed high antifibrinolysin titres. Streptococcus infections produced an antistreptolysin response more frequently than an antifibrinolysin response. Of the 232 hospitalized soldiers studied with exudative tonsilitis or pharyngitis from whom  $\beta$ -hemolytic streptococci were isolated by throat culture, 151 showed an increase in antistreptolysin antibodies, whereas in only 68 was there an increase in antifibrinolysin titre.

Thirty-seven per cent of patients with streptococcal tonsilitis developed antifibrinolysin antibodies during convalescence. In this series of patients, with streptococcal infections of the throat, those showing an increase of antistreptolysin also showed an increase in the antifibrinolysin titer. Although the majority of these patients with *proved* streptococcal infection were mildly ill, there were no obvious differences in the severity of illness of those patients who did or did not exhibit an increase in the antifibrinolytic titer. It was found that, in general, those streptococci which stimulate antifibrinolysin formation *in vivo*, produce large amounts *in vitro*, while those streptococci that produce small amounts of fibrinolytic factor generally failed to stimulate antibody formation.

## 2. Postulates on the Role of the Bacterial Fibrinolytic Factor

Garner and Tillett (1934a) obtained a preparation of fibrinolytic factor from broth filtrate of streptococcal culture by precipitating with three volumes of alcohol, or by adsorption on alumina and elution by phosphate buffer. The preparation was relatively heat resistant, and stable in the dry form for over a year's period. The optimal temperature of activity was within the range of 35° to 45°C., and the optimal pH of activity was 7.3. The activity of the factor was rapidly destroyed by trypsin and by papain. The factor was incapable of digesting casein, gelatin or peptone. They claimed that they had recovered bacterial factor from a reaction mixture which had undergone lysis and then demonstrated its activity on a new sample of fibrin. *There is no information concerning the question whether or not the recovered sample contained the active serum-protease. This protease, if present, could have been responsible for the lysis of the new sample of fibrin.* In the absence of specific information concerning this question the original claim of Garner and Tillett (1934a) (based on this experiment) that bacterial fibrinolytic factor behaves like an enzyme requires verification.

Christensen (1945, 1946) using a partially purified fibrinolytic factor, found that his preparation was only slowly destroyed by heating at 100°C. at pH 7.4. It was readily destroyed by treatment with trypsin and pepsin, indicating that the activity was associated with a protein. This factor *per se* lacking proteolytic activity when added to protein solutions as substrates, raises the question as to how it exercises its effect on the lysis of a fibrin clot. Milstone (1941) had made the important observation that human serum and plasma contains a "lytic factor" which is essential for the lysis of fibrin by streptococcal fibrinolytic factor. If fibrin is free from traces of serum or plasma, the streptococcal factor has no effect on fibrin clot. Such fibrin was rendered susceptible to the action of streptococcal factor, however, by the addition of "lytic factor" present in the euglobulin fraction of normal human serum. Rabbit fibrin which has been reported to be insensitive to the action of the streptococcal factor, likewise was dissolved when treated with human "lytic factor."



Tagnon (1942), Tagnon, *et al.* (1942), Kaplan, *et al.* (1942) showed that chloroform-activated serum acting on fibrinogen, fibrin, gelatin, and casein produces proteolytic digestion indicated by the progressive formation of non-protein nitrogen from these substrates. Christensen (1945) reported that lysis of fibrin clot taking place with the participation of bacterial factor is likewise associated with proteolysis. Garner and Tillett (1934b) had reported that if fibrinogen is incubated with bacterial fibrinolytic factor for a brief period, it can no longer be changed to fibrin upon the addition of thrombin. Nevertheless they concluded that the degradation of the fibrinogen molecule is not great. Jablonowitz (1938) reported that bacterial factor changes the specificity of the fibrinogen, as evidenced by the change in the quantity of the precipitates when mixtures of bacterial factor and fibrinogen are tested with antifibrinogen serum. In the light of recent studies it is apparent that Garner and Tillett, and Jablonowitz were experimenting with fibrinogen contaminated with serum-protease liberated during the action of bacterial factor on fibrinogen.

From the standpoint of understanding the role of bacterial fibrinolytic factor in the lysis of fibrin formed from serum, plasma or impure fibrinogen several points should be emphasized. It is to be noted that a plasma clot or fibrin is dissolved, in the absence of bacterial factor, when serum or plasma are treated by organic solvents such as chloroform, ether, alcohol or acetone. Also treatment with concentrated solution of sodium chloride, adsorption with talc or kieselguhr, simple dialysis, or merely standing can activate the inert fibrinolytic or proteolytic enzyme in serum.

The liberation of fibrinolysis from its inactive complex in serum was reported by Ungar and Mist (1949) under the following conditions: (a) by adding the specific antigen to serum from sensitized guinea pigs, and (b) by mixing normal guinea pig serum with peptone, agar, hyaluronic acid, chondroitin sulfuric acid, glycogen, pneumococcal polysaccharide, and heparin. In other words, treatments with these non-specific means produce the active enzyme from whatever state it may have been present in normal serum. It is interesting to keep in mind the fact that streptococcal fibrinolytic factor produces the same effect on inert serum as, for example, a simple dialysis. It is difficult to conceive that, under these conditions, the inert state of the serum enzyme molecule *per se* undergoes a chemical structural change to yield an enzy-



matically active new molecule. It may here be suggested that the role of streptococcal factor in the lytic process may represent a simple process or splitting of the already structurally complete proteolytic enzyme from an easily dissociable combination with a substance which blocks the enzymatic activity.

Many years ago, Nolf (1908) observed that fibrinolytic activity was obtained when recalcified plasma was shaken with chloroform. Several investigators (Jobling and Peterson, 1914; Minot, 1915; Yamakowa, 1918) observed that when serum was treated with chloroform or other organic solvents the anti-trypsin (or antithrombin) was removed, producing autodigestion or proteolytic action (Yamakowa, 1918). These observations have recently been reinvestigated and considerably extended (Patek and Taylor, 1937; Tagnon, 1942; Tagnon, *et al.*, 1942; Kaplan, *et al.*, 1942; Kaplan, 1944). These investigators reported that when platelet-free normal human plasma, claimed to be free from active calcium ion, is treated with chloroform, the euglobulin fraction of the plasma demonstrates the property of lyzing both fibrin and fibrinogen. At low concentration of the chloroform-activated globulin, clotting of fibrinogen occurred, while at higher concentration the clotting was followed by fibrinolysis, and at still higher concentrations fibrinogenolysis occurred with no clotting. Chloroform treated plasma also digested gelatin, casein and hemoglobin, forming non-protein nitrogen (Kaplan, *et al.*, 1942; Kaplan, 1944). They stated that the activity of the chloroform treated plasma resembles, in some respects, that of trypsin in its action on plasma, fibrinogen and prothrombin by causing the clotting of the oxalated blood and fibrinogen solution followed by lysis of the fibrin clot.

The above findings are helpful for the understanding of the nature and the manner with which the so-called bacterial, more specifically streptococcal, fibrinolysins function. In the absence of serum lytic factor, as Milstone (1941) first demonstrated, bacterial factor has no effect on the lysis of fibrin. Christensen (1945) proposed that bacterial factor catalytically activates the serum lytic factor.

Christensen and MacLeod (1945) referred to the already observed facts that certain non-specific agents, such as organic solvents, acid precipitation, treatment with urea, benzoate, thiocyanate and cresols, activate serum lytic factor. Schmitz (1937) attributed the activation of lytic factor resulting from treatments with alumina gel, or dialysis in

dilute acetic acid, to the splitting of the inhibitor-enzyme complex, the inhibitor being adsorbable, acid soluble, and dialyzable. Despite the similarity between the above cited activations and that by streptococcal factor, Christensen and MacLeod are of the belief that streptococcal factor functions in a manner comparable to the action of enterokinase (Kunitz, 1938) on trypsinogen. They reported that, in the absence of large amounts of serum inhibitor, the activation of the serum lytic factor by streptococcal factor approaches that of a first order reaction, suggesting to them a catalytic type of activation. The activated serum protease is inhibited by serum inhibitor or crystalline trypsin inhibitor (from pancreas). However, serum protease required very much more trypsin inhibitor than required by trypsin. Unlike that of equimolar trypsin-inhibitor complex several molecules of inhibitor were required by serum protease before inhibition was complete. This inhibition, however, was not influenced by streptococcal factor, which was interpreted to indicate that serum protease is not identical with pancreatic trypsin. Since an excess amount of streptococcal factor did not increase the activity of the serum protease which was partially inhibited by crystalline trypsin inhibitor, and since the streptococcal factor is capable of converting the inactive serum lytic factor into a proteolytic enzyme, it was concluded that the inactive state of serum protease is not due to a combination of the protease with serum inhibitor but to some other mechanism.

In a later study, Christensen (1946) compared the activation of serum lytic factor with chloroform and streptococcal factor. In this study, he introduced the term *plasminogen* for the inactive state of serum lytic factor and *plasmin* for the active state. Reaffirming his claim, he stated that the activation of plasminogen by streptococcal factor is a catalytic, and that by chloroform is a non-catalytic process.

The above claims that streptococcal factor functions as kinase is contradicted by the finding that the active proteinase can be liberated by drying the whole plasma. Shinowara (1947) reported that dried whole plasma (and its fractions prepared by low-temperature-ethanol procedure) contained a high degree of proteolytic activity. The dried plasma and its fractions were not subjected to previous treatment with kinase or chloroform or any other agents. This observation would indicate that the union between the inhibitor substance and the proteinase is dis-

rupted by the drying process, and the inhibitor is eliminated by the low-temperature ethanol fractionation.

a. **Comments on the Possible Nature of the Role of Bacterial Fibrinolytic Factor.** The above cited important studies by various investigators no doubt have very much broadened our knowledge of the process of fibrinolysis. There appear, however, certain questions which need to be investigated to further our understanding of this process. One would like to know the chemical nature and the properties of the substance which apparently is present in serum and keeps the serum protease in an inactive state. This substance is apparently readily eliminated by treatment with organic solvents such as chloroform. Is this a lipid-like substance comparable to thromboplastin or one of its degradation products of non-protein nature, or a polypeptide comparable to the trypsin inhibitor obtained from pancreas? The inhibition of activated serum protease by pancreatic trypsin inhibitor does not necessarily mean that the inhibitor component of the inactive serum protease complex is chemically identical with the pancreatic trypsin inhibitor. The inability of the bacterial factor to influence the inhibition of serum protease resulting from combination with pancreatic trypsin inhibitor may indicate that the inhibitor component of the inactive state of serum protease is different from the trypsin inhibitor. The activation of serum lytic factor by organic fat solvents suggests the possibility that this inhibitor serum component is a lipid-like substance. Does the material recovered from organic solvents exercise inhibition when returned to the activated serum protease? If it inhibits, is this inhibition antagonized, or destroyed by streptococcal factor?

The possibility that a lipid-like substance is removed by solvents, etc., is strengthened by an observation (Tagnon, 1942) that chloroform-activated serum loses its fibrinolytic power when treated with thromboplastin. Tagnon reported that oxalated plasma contains antilytic substances. He demonstrated that thromboplastin exercises a powerful antilytic effect. A reaction system containing chloroform activated serum enzyme was capable of digesting fibrinogen, without producing fibrin clot formation. In the presence of a small amount of thromboplastin the same system caused the clotting of fibrinogen but no lysis of fibrin clot took place. That this inhibition may be considered as the result of a direct action of thromboplastin on the fibrinolytic

serum protease may further be strengthened by consideration of the interrelation of the following observations:

- (a) Prothrombin + thromboplastin +  $\text{Ca}^{++} \rightarrow$  thrombin;
- (b) Prothrombin does not inhibit fibrinolysin, but is destroyed by it;
- (c) Thrombin has no demonstrable fibrinolytic activity and is resistant to the proteolytic action of serum protease (Loomis, *et al.* 1947); and
- (d) Thromboplastin lacks any demonstrable trypsin-like enzymatic activity (Chargaff, 1945).

Does thromboplastin combine with the activated serum protease causing its inactivation? Does such a reaction account for the inactive state of the serum lytic factor? Or does thromboplastin combine with the bacterial fibrinolytic factor preventing its role of activating the serum lytic factor? In this latter case the role of bacterial factor will be equivalent to the action of organic solvents whereby the inhibitor factor (or a lipid-like substance) is eliminated by its extraction with chloroform.

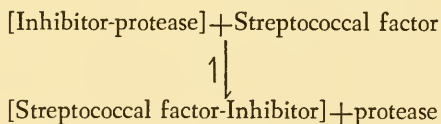
Evidence that a lipid-like substance keeps the normal serum proteinase in an inactive state is suggested by the observations of Jobling and Petersen (1914). They advanced evidence that the normal antiproteinase action of serum depends on the serum lipids. They were able to decrease the anti-proteolytic action of, or produce proteolytic activity in serum, by extracting the lipids with fat solvents. They blocked the proteolytic activity of the extracted serum by replacing the lipids. The anti-proteolytic lipids contained unsaturated carbon bonds; by saturating the double bonds with iodine they found the lipids to have lost their anti-proteolytic activity. Soaps of saturated fatty acids were found to be unable to function as anti-proteolytic factor. Unsaturated soaps neutralized the proteolytic activity. The unsaturated lipids (serum-anti-trypsin) could be adsorbed from serum by kaolin, starch, agar, and bacteria, rendering the serum proteolytically active. It is interesting to note that bacteria treated with serum or oil did not adsorb serum lipids. Bacteria were rendered resistant to the action of serum proteinase by the adsorption of lipids. These observations would seem to supply considerable support to the idea that streptococcal factor is combining with the anti-proteolytic lipids and is thus rendering the serum proteolytically active. However, for another possible mechanism for the activation of serum the reader is referred to a suggestion which is discussed further below.



The bacterial factor merits a further study from another angle. It is a protein and is destroyed by the proteolytic enzymes trypsin, pepsin or papain.

Rothbard and Todd (1948) reported that the streptococcal proteinase digests both the fibrinolytic factor ("Streptokinase") and M protein antigen. The latter two components are not, therefore, usually found in the same cultures with proteinase. Chloroform activated serum protease has been found to digest gelatin, casein, hemoglobin, fibrinogen and fibrin, and shows, in some respects, similarity to trypsin. Does the fibrinolytic serum protease digest the streptococcal factor immediately following the completion of its activating role? Or does the streptococcal factor combining with the inhibitor component of the inactive serum protease complex remain inactive and insensitive to the proteolytic action of the activated enzyme? These questions may merit consideration for a satisfactory evaluation of the results of kinetic studies in fibrinolytic and activation processes.

On this basis, the activation of the inactive serum-protease by streptococcal factor would be governed by a stoichiometrical relationship and not by a catalytic activation. That is, the streptococcal factor would stoichiometrically combine with the inhibitor substance, possibly a lipid, and thereby set free the active proteinase. In other words, the affinity of the inhibitor substance for the streptococcal factor would be greater than its affinity for the serum protease:



The recent observations by Ratnoff (1948) would seem to be most significant in this connection. Reinvestigating the problem of whether or not the activation of serum by streptococcal factor is catalytic, he arrives at the conclusion that his data indicate that "in the presence of some substance altered by chloroform or heat, the activation of plasma proteolytic enzyme by streptococcal factor behaved as if it involved a stoichiometric reaction. Streptococcal factor seemed to react in molecular proportions with a substance in plasma euglobulin which limited the activation of plasma proteolytic enzyme." In disagreement with the claim of Christensen, *et al.*, ascribing a catalytic role for streptococcal factor, Ratnoff finds that the proteolytic activity evoked by this factor



acting on plasma globulin preparations not treated with heat or chloroform is an hyperbolic function of the concentration of the streptococcal factor. This is stated to be compatible with the law of mass action.

**b. A Suggestion as to the Possible Lipolytic Role of Streptococcal Factor.** It may be that the role of streptococcal factor in the liberation of serum protease from its inactive complex truly involves an enzyme action. Integrating the activating effect of organic solvents, suggesting that a lipid-like inhibitor substance is thereby eliminated, and the inhibition of the fibrinolytic activity of chloroform-activated serum lytic enzyme by thromboplastin with the activating effect produced by streptococcal factor on inactive serum lytic factor one may be permitted to reason that the critical substance requiring elimination is a lipid-like substance. This effect may suggest that the bacterial fibrinolytic factor is, perhaps, a *lipolytic* (lipase, lecithinase) enzyme. Such an enzyme would be capable of digesting the lipid component of serum lytic factor, thus liberating the active enzyme, an effect which would be comparable to the elimination of the same lipid-like component by physical action of organic solvents, etc. Such a finding would throw a new light on the subject and harmonize various observations.

In connection with the possible lipase-like nature of streptococcal factor it may be of interest to recall that both the streptococcal factor and bacterial lecithinases are relatively thermostable (see Garner and Tillett, 1934a; Dart, 1936; Reed, *et al.* 1943; Christensen, 1945; MacFarlane and Knight, 1941). The question of whether or not the comparable thermostability of the streptococcal factor and bacterial lecithinases is suggestive of a similarity of activity may merit consideration.

## G. MECHANISM OF FIBRIN AND MILK CLOT FORMATION

### 1. Enzymes Responsible for the Formation of Plasma Clot

**a. Comment on the Use of the Term Coagulation.** The conversion of plasma (or blood) or fibrinogen into a clot or fibrin has been called *coagulation*, *clotting* or *fibrin clot formation*. One is struck by the wide use of the term coagulation in this sense. The term coagulation as defined stands for various chemically and physiologically non-related processes, and for the formation of jelly-like soft masses or clots from

proteins by the action of the enzyme thrombin. Coagulation by heat and chemical agents involves denaturation reactions. Clotting of fibrinogen by thrombin is a specific enzyme reaction involving none of the denaturation reactions. Coagulation by denaturation is "any non-proteolytic modification of the unique structure of a native protein, giving rise to definite changes in chemical, physical, or biological properties" (Neurath, *et al.* 1944). Fibrin clot formation by the action of thrombin, in contrast, is an enzyme process involving the linkage of inter-molecular bonds leading to the formation of fibrin of higher structure from the fibrinogen molecules. Coagulation of a protein by denaturation, as we know now, presents hydrophobic compact protein aggregates devoid of elastic and other physical properties characteristic of fibrin clots. It would, therefore, be more precise to avoid the use of the non-specific term coagulation in describing the conversion of fibrinogen specifically into fibrin clot.

**b. Factors Responsible for the Formation of Plasma Clot.** The available information regarding the mechanism of blood clotting instructs us that the blood plasma factor, *prothrombin*, is activated by *calcium ion* and platelets, or tissue extracts, or simply by purified *thromboplastin* (lipo-protein) to form *thrombin*, which catalyzes the conversion of fibrinogen in plasma into fibrin clot. Other substances of animal and vegetable origin have been reported to exercise thrombic activity. Eagle and Harris (1937) reported that trypsin, which has no direct clotting action on purified fibrinogen, converts prothrombin to thrombin; and this thrombin then acts on blood fibrinogen to form fibrin. With an excess of trypsin, no thrombin formation was demonstrated, because of the digestion of prothrombin, thrombin, or both. They also reported that papain, unlike trypsin, did not activate prothrombin to thrombin but acted directly on fibrinogen in changing it to fibrin. The reaction between trypsin and prothrombin, as well as the action of papain on fibrinogen in changing it to fibrin, according to them, is independent of calcium ion, platelets or tissue extracts. Trypsin and the Ca-tissue (or Ca-platelet) system were looked upon as mutually supplementary. Both systems affect the same substrate, prothrombin, to form as end products clots which are qualitatively indistinguishable.\*

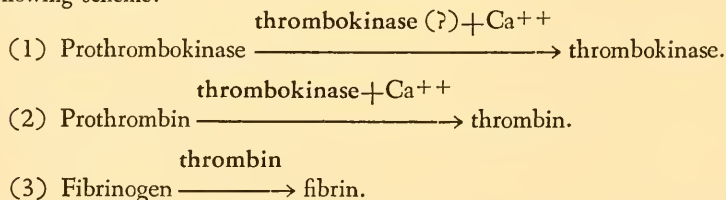
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\*Studies dealing with the mechanism of blood clotting, and of the interaction of various factors leading to or inhibiting blood clotting, constitute a vast array of

c. **Enzyme Nature of Thrombin.** Schmidt (1892, 1895), one of the earliest investigators of the problem of plasma clotting, termed thrombin a fibrin ferment, and considered it to be a proteolytic enzyme which split fibrinogen, forming fibrin. That thrombin is an enzyme has been established, after many controversies, by the application of rigid criteria of catalysis. It has been found that the amount of fibrin formed is independent of the amount of thrombin present in a system, over a wide range. Eagle (1935) reported that a purified preparation of thrombin clotted over 200 times its own weight of fibrinogen. On the other hand, Ferry and Morrison (1947) calculated that thrombin is capable of converting, over 100,000 times its own weight of fibrinogen to fibrin. These findings show decisively that the role of thrombin in the conversion of fibrinogen into fibrin is one of enzyme catalysis.

d. **Conversion of Fibrinogen into Fibrin.** Measured by double refraction flow, viscosity and osmotic pressure, fibrinogen has been reported to possess dimension of about from  $35 \times 700$  to  $33 \times 900$  Å and a molecular weight of the order of 300,000 to 500,000 (Edsall, Ferry, and Armstrong, Jr., 1944; Ferry and Morrison, 1947). Measuring the opacity, modulus of rigidity, friability, syneresis and other mechanical properties of fibrin clot, Ferry and Morrison (1947) and Hawn and Porter (1947) studied the mechanism of the conversion of fibrinogen into fibrin. These measurements were intended to determine: (a) the

literature. The views expressed on certain aspects of these questions are of controversial nature. In presenting the above view it is not intended to pass over the others without due notice. The reader is referred to a treatise by Quick (1942) for a comprehensive discussion of the various aspects of the subject, to an article by Ferguson (1943), and to a critical review by Chargaff (1945). Milstone (1948) summarized an extensive study on the three stage analysis of blood clotting with the following scheme:



According to him all three reactions are enzymatic, and calcium ion conditions the reactions. According to Quick (1947) the reactions that bring about the production of thrombin from the prothrombin complex, thromboplastin and calcium, are chemical and not enzymatic. Thromboplastin acts stoichiometrically, and the relation of calcium concentration to thrombin production is also stoichiometric. According to Loomis and Seegers (1946) there is no evidence of the existence of a prothrombin complex; it is a homogeneous protein.

nature of the chemical bonds which link fibrinogen units together; and, (b) the geometrical arrangement of the fibrinogen units in the fibrin structure.

In very dilute solution, the mode of junction of fibrinogen units in forming fibrin clot was found to be primarily an end-to-end tridimensional polymerization, indicated by the fact that a volume fraction of as little as 0.02 to 0.04 g/liter of fibrinogen solution sufficed for gelation, which meant that the ratio of length to diameter of the network strand is very great. They characterized two extreme types of fibrin clot, "coarse" and "fine." In "fine" clot, the structural unit was small, transparent, elastic, friable and non-synerizing, and was assumed to be a single chain of fibrinogen molecules, joined end-to-end with fairly strong crosslinks. The fact that fibrin was found to be insoluble in reagents such as urea and potassium thiocyanate, which often dissociate protein structures, was offered as evidence that at least some of the crosslinks are primary chemical bonds. Secondary bonding was not expected, for the conditions used for the formation of "fine" clot were not conducive. They assumed that the properties of the clot are similar to those of a swollen gel of vulcanized rubber. They also considered the opening of a single fibrinogen molecule to form an extended polypeptide chain as observed in the denaturation of corpuscular proteins as another mechanism for providing a strand with an axial ratio of several hundred. It was held unlikely, however, for the reason that the "fine" clot structure is stable. Denatured corpuscular proteins are generally so hydrophobic that they aggregate and form compact precipitates unless held in solution by urea, strong acid or alkali or detergents. By contrast, a fine structure of fibrin clot was stated to be well-dispersed at pH 7; it was rigid, but the strands showed no tendency to roll up and the opacity studies showed no great tendency to lateral aggregation.

The permeability of the film to small protein molecules was studied by Ferry and Morrison (1947b). Among these, the permeability of the serum proteolytic enzyme plasmin (fibrinolysin) was determined by measuring the rate of fibrin digestion. The weight of fibrin digested was proportional to the time, and the rate of digestion increased with increasing concentration of enzyme. Moreover, the rate of fibrin destruction was roughly proportional to the thickness of the film, which indicated to them that the enzyme penetrates the structure and that digestion proceeds throughout the volume of the fibrin film. If the film



were impermeable and digestion limited to the surface, the rate of destruction should be independent of thickness. No determinations were reported concerning the nature of the products of proteolytic digestion of fibrin.

## 2. Chemical Reactions Involving $-S-S-$ Linkages in the Conversion of Fibrinogen to Fibrin

Analyzing the conversion of fibrinogen to fibrin from the standpoint of equilibrium of chemical reactions one would find that the formation of insoluble fibrin from a solution of fibrinogen is another example of those reactions which proceed uninterruptedly to completion and appear to be irreversible; in these reactions the initial substances are exhausted. On the other hand, the reversible reactions, at equilibrium, remain more or less incomplete due to the reverse action of the reaction products yielding the initial substances. In the former, the reaction products are either insoluble or are of gaseous nature whereby they are eliminated from the field of reaction either by forming insoluble precipitates or by escaping, e.g., the formation of very sparingly soluble silver chloride, or the formation of hydrogen from metallic sodium and water, or the formation of insoluble fibrin from soluble fibrinogen.

The formation of fibrin from fibrinogen as discussed above possesses features which might seem to be comparable with the synthesis of higher insoluble polypeptides from simpler water soluble ones by the catalytic action of papain. After discussing various aspects of reactions at equilibrium, Bergmann and Fruton (1941) described numerous reactions of this type. For example, when cysteine-activated papain was added, at 40°C., to a solution containing equivalent amounts of acetyl-dl-phenylalanylglycine and aniline, after several minutes a copious crystallization of acetyl-l-phenylalanylglycine anilide was formed. The crystallization of anilide will disturb the equilibrium; the reaction will continue to proceed to the right until virtually all of the initial reactants are converted into anilide.

This simple one-step synthesis of sparingly soluble polypeptides may in principle appear to be analogous to the formation of fibrin from fibrinogen by the action of papain as a proteolytic process. The question of whether or not papain brings about this reaction as a synthetic



proteinase converting fibrinogen into insoluble fibrin with very little, if any, proteolytic action on the fibrinogen molecule, seems to require consideration. Since theoretically *all actions are fundamentally reversible*, and also since a catalyst accelerates both directions of a reaction at equal rate to attain equilibrium, it may, therefore, at first, seem reasonable to assume that papain functions as a synthetic as well as a digestive enzyme. If we assume that thrombin catalyzes the conversion of fibrinogen to fibrin in a similar manner, it would necessitate that we accord to thrombin the rôle of a specific proteinase. In view of the fact, however, that thrombin has not been shown to possess proteolytic activity, it would seem necessary that the action of this enzyme be explained in another manner.

In connection with the nature of the primary chemical bonds involved in the conversion of fibrinogen to fibrin a discussion by Chargaff (1945), and, particularly, a study by Lyons (1945), are of interest. In a critical review, Chargaff pointed out that fibrinogen solution free from prothrombin forms clots when acted upon by a number of simple organic substances such as sodium N-chloro-p-toluenesulfonamide or chloramine T, potassium 1,4-naphthoquinone-2-sulfonate, sodium 1,2-naphthoquinone-4-sulfonate, ninhydrin (1,2,3-indenetrionehydrate) and, much less markedly, alloxan and salicylaldehyde. The clots were coherent and rapidly retracting. Discussing various reactions as possible mechanisms or alterations in the fibrinogen molecule forming the fibrin structure, he stated that the activity of these clotting agents was found to parallel their ability to decarboxylate amino acids of the fibrinogen molecule. The possibility of oxidation of susceptible groupings, such as sulfhydryl, was also considered. From the fact that reducing substances, such as sodium bisulfite and glutathione, inhibited the action of the clotting agents, an oxidative reaction appeared to be responsible for clotting. Though there was some indication of the evolution of carbon dioxide from the action of thrombin on fibrinogen in an oxygen-free atmosphere, Chargaff was non-committal regarding the relationship of these reactions to the mechanism of true clot formation. The question of whether the clots formed by the action of organic substances were comparable to normal fibrin, or were similarly susceptible to the fibrinolytic action of serum lytic factor was not discussed.

Chargaff and Ziff (1941) reported that "ninhydrin" would clot

fibrinogen, and Chargaff and Bendich (1943) found that other naphthoquinone derivatives were capable of clotting fibrinogen, but they denied that vitamin K (2-methyl-3-phytyl-1,4-naphthoquinone) had this property for the fact that two sulfonic acid derivatives of naphthoquinone which have vitamin K activity, did not clot fibrinogen. The quinone group is characteristic of vitamin K<sub>1</sub>. Lyons (1945) reported that 2-methyl-1,4-naphthoquinone, which is reported to be two to three times as potent as vitamin K<sub>1</sub>, can form a gel with *specifically prepared fibrinogen* and that this gel is indistinguishable microscopically from a thrombin clot. He believes that 2-methyl-1,4-naphthoquinone acts by oxidizing the thiol groups of fibrinogen prepared from aged, sterile plasma which has had a preliminary treatment with calcium, producing an immediate gel.

Polarographic estimations performed at intervals during clotting suggested to him that initially *fresh fibrinogen* (A) contains many blocked protein-SH groups which are quickly converted by thrombin to protein-SH. The protein-SH form of fibrinogen can be prepared from aged citrated plasma, and this type of fibrinogen (B) can be immediately converted by minute amounts of 2-methyl-1,4-naphthoquinone to form protein-S-S-protein giving a clot. The clot thus formed is indistinguishable microscopically from the clot formed with thrombin, having a typical gel structure.

Lyons believes that vitamin K is an integral and functional part of the prothrombin molecule. The presence of small amounts of naphthoquinone derivative in thrombin was suggested by two color reactions which were applied to a tryptic digest of thrombin (naphthoquinone gives a green color with 2:4-dinitrophenylhydrazine and develops a light blue color with ethylcyano-acetate).

Lyons reports that both fibrinogen A and B completely lose their clotting properties when treated with an organic mercurial (merthiolate, sodium ethyl mercuri-thiosalicylate), since they block the oxidation of sulfhydryl groups by combining with them. Fibrin is readily soluble in *sodium sulphide* at pH 7.5, suggesting the presence of disulfide linkages which are disrupted by sodium sulfide, thus:



The reaction of thrombin with excess thiol compound, sodium thioglycollate, was irreversible as attempts to regenerate active throm-

bin failed. Sodium cyanide and arsenates inhibited clotting. Quantitative determinations were carried out by the use of the reaction  $2\text{NaN}_3 + \text{I}_2 \rightarrow 2\text{NaI} + 3\text{N}_2$  which is catalyzed by -SH and blocked-SH groups and showed that all reactions and properties of fibrinogen A are typical of the blocked protein-SH which is later converted to protein-SH or fibrinogen B. The latter is oxidized by a thrombin component (possibly a naphthoquinone complex) to a protein-S-S-protein form. A large number of these linkages combining fibrinogen B molecules would give the typical fibrin structure.

On the basis of the results discussed above, it would seem that thrombin exposes the blocked protein-SH in fibrinogen molecules in fresh plasma, and the naphthoquinone, or vitamin K-like component of thrombin oxidizes the exposed protein-SH to a R-S-S-R structure yielding the fibrin structure. Fibrinogen prepared from aged plasma which possesses the protein-SH groups already in an exposed form, can directly be oxidized to the disulfide linkage, yielding the fibrin structure.

The above interpretation of the mechanism of the conversion of fibrinogen to fibrin structure may explain also the unique role of papain among the proteolytic enzymes in clotting fibrinogen molecules. It has been known that papain is activated by hydrocyanic acid, hydrogen sulfide, glutathione, cysteine, sodium thiosulfate and other reducing agents. It is inactivated by hydrogen peroxide and iodoacetic acid. In an active state papain may be considered as a SH-protein. In an inactive state it would contain the -S-S- linkage which can oxidize -SH groups of fibrinogen molecules yielding the fibrin structure. Thus the clotting of fibrinogen by papain may be referred to its oxidative-reductive properties through its thiol groupings. Papain may also bring out the blocked -SH groupings in the fibrinogen molecules for their conversion to -S-S- linkages, yielding the fibrin structure.

### 3. Antibody Against Thrombin. Neutralization of Thrombin of Rabbit Serum by Anti-Rabbit Guinea Pig Immune Serum

Bordet and Gengou (1901) showed that a neutralizing antibody against the enzyme of rabbit blood responsible for clotting of the blood or plasma could be produced in guinea pigs.

Immune guinea pigs' serum against the blood clotting enzyme was prepared by three injections of 5 ml. of fresh rabbit serum at about 8-day intervals. The other guinea pigs received rabbit plasma which

TABLE XV

*Inhibition of Plasma Clotting Enzyme of Rabbit Serum by Anti-Rabbit Guinea Pig Serum*

Series A	Results
(a) 0.1 ml. of unheated rabbit serum +0.3 ml. of avian plasma	Clotting of avian plasma within 15 to 30 minutes
(b) 0.1 ml. of unheated normal guinea pig serum +0.3 ml. of avian plasma	Clotting of avian plasma within 15 to 30 minutes
(c) 0.1 ml. of unheated immune guinea pig serum +0.3 ml. of avian plasma	Clotting of avian plasma within 15 to 30 minutes
Series B	
(a) 0.6 ml. of heated rabbit serum +0.3 ml. of avian plasma	Avian plasma indefinitely liquid
(b) 0.6 ml. of normal heated guinea pig serum +0.3 ml. of avian plasma	Avian plasma indefinitely liquid
(c) 0.6 ml. of heated immune guinea pig serum +0.3 ml. of avian plasma	Avian plasma indefinitely liquid
Series C	
(a) 0.1 ml. of unheated rabbit serum +0.6 ml. of heated normal guinea pig serum +0.3 ml. of avian plasma	Clotting a few minutes slower than in Series A
(b) 0.1 ml. of unheated normal guinea pig serum +0.6 ml. of heated normal guinea pig serum +0.3 ml. of avian plasma	Clotting a few minutes slower than in Series A
(c) 0.1 ml. of unheated immune guinea pig serum +0.6 ml. of heated normal guinea pig serum +0.3 ml. of avian plasma	Clotting a few minutes slower than in Series A

Series D	Results
(a) 0.1 ml. of unheated rabbit serum +0.6 ml. of heated immune guinea pig serum +0.3 ml. of avian plasma	Remains <i>indefinitely liquid</i>
(b) 0.1 ml. of unheated normal guinea pig serum +0.6 ml. of heated immune guinea pig serum +0.3 ml. of avian plasma	Clots, though at a slower rate.
(c) 0.1 ml. of unheated immune guinea pig serum +0.6 ml. of heated immune guinea pig serum +0.3 ml. of avian plasma	Clotting complete within 90 minutes without pencillia
Series E	
(a) 0.1 ml. of unheated normal rabbit serum +0.6 ml. of heated normal rabbit serum +0.3 ml. of avian plasma	Clotting immediate
(b) 0.1 ml. of unheated normal guinea pig serum +0.6 ml. of heated normal rabbit serum +0.3 ml. of avian plasma	Clotting immediate
(c) 0.1 ml. of unheated immune guinea pig serum +0.6 ml. of heated normal rabbit serum +0.3 ml. of avian plasma	Clotting immediate

was prepared by centrifuging oxalated rabbit blood. The oxalated plasma was treated with a calculated amount of calcium chloride and a dose of 5 ml. was then subcutaneously injected into guinea pigs. The oxalated plasma thus treated with calcium chloride did not clot until after a period of 10–12 minutes. Injections were carried out before the lapse of this period. Using the above technique it was possible to inject the animals with the fibrinogen mixed with fibrin enzyme (thrombin) without causing clotting.

The immunized guinea pigs were bled 12 days after the last injection. The immune serum was found to neutralize the thrombin activity of fresh rabbit blood or serum. Non-clotting avian plasma with high fibrinogen content was used as the substrate in inhibition or neutralization experiments.

*The Avian Plasma.* Bordet and Gengou found that avian plasma clots very slowly if the bleeding is carried out without contaminating



the blood with the products of injured tissue. They found that this plasma rich in fibrinogen constituted an excellent substrate for "ferment-fibrin" or thrombin as we know it now. It clots rapidly when treated with a trace of fresh serum of different species of animals, guinea pig, rabbit, sheep, dog, etc. The serum of birds clotted this plasma very slowly because of the low clotting enzyme (thrombin) content of bird's blood.

The thrombin activity of sera is destroyed when heated at  $58.5^{\circ}\text{C}$ . for 45 minutes. When a mixture of fresh rabbit serum and normal guinea pig serum (inactivated at  $58.5^{\circ}\text{C}$ .) was added to avian plasma the latter clotted because of the presence of enzyme in the fresh rabbit serum. In contrast, a mixture of fresh rabbit serum with inactivated anti-rabbit immune guinea pig serum was incapable of clotting the same avian plasma. This showed that anti-rabbit immune guinea pig serum neutralized the enzyme in the fresh rabbit serum. The presence of an anti-enzyme antibody in immune guinea pig serum resistant to a temperature of  $58.5^{\circ}\text{C}$ . was thus demonstrated in the above, as well as in the following series of experiments.

From Table XV it is to be seen that the enzyme present in fresh rabbit serum and responsible for the clotting of the avian plasma was specifically inhibited by homologous immune guinea pig serum. The complete inhibition of the enzyme present in one volume of fresh rabbit serum required six volumes of immune guinea pig serum. If instead, three volumes of immune serum were used the inhibition of the enzyme was marked but not complete.

The immune guinea pig serum had no inhibitory effect on the enzyme of fresh normal serum of dog or sheep, showing a high degree of specificity.

Immunization of the rabbit with guinea pig serum produced in the rabbit antibodies which likewise neutralized the plasma clotting enzyme of the guinea pig serum. It had no inhibitory effect on the enzyme of normal rabbit serum in the same way that anti-rabbit immune guinea pig serum was ineffective against the enzyme of normal guinea pig serum.

#### 4. Antibody Against the Plasma Clotting Enzymes of Snake Venom

a. **Enzymic Nature of Blood Clotting Activity of Snake Venoms.** Schmidt (1892, 1895), one of the earliest men to work on the plasma-clotting problem, termed thrombin a fibrin-ferment.

Eagle studied (1937, 1939) several snake venoms with regard to their blood clotting properties. Certain venoms were shown to inhibit the clotting process when added to blood *in vivo* and *in vitro*, a few had no significant effect, and a large number exercised marked clotting action on whole blood, plasma, or fibrinogen.

Trypsin, like Ca-platelets (or Ca-tissue), catalyzes the transformation of prothrombin to thrombin, and papain, like thrombin, catalyzes the transformation of fibrinogen to fibrin. Eagle investigated the question as to whether the clotting action of snake venoms depends on their enzyme content, and whether these venoms are of two types. The question formulated by him was as follows:

(a) Is there one type which, like trypsin and like the Ca-platelet system, acts on prothrombin to form thrombin; and

(b) another which, like papain and thrombin, acts directly on fibrinogen to form fibrin. The experimental data supported his assumptions.

*Fibrinogen.* The purified fibrinogen used in these experiments did not clot on the addition of calcium and lung extract, but promptly formed clot by thrombin.

*Prothrombin.* The crude prothrombin preparation was freed from fibrinogen. The isotonic solution of prothrombin preparation rapidly evolved thrombin in large amounts on the addition of 0.5 volume of 1 per cent calcium chloride and cephalin (or lung extract). The addition of calcium alone had either no demonstrable effect, or caused a very slow elaboration of minute quantities of thrombin, less than 2 per cent of the amount elaborated from the same prothrombin in the presence of an adequate amount of cephalin or tissue derivatives.

b. **The Clotting of Fibrinogen by Snake Venoms.** Eight of the seventeen venoms tested exercised active clotting effects, and one (*Crotalus horridus*) yielded variable results.

Seven of the nine venoms which clotted plasma also clotted purified

fibrinogen. These seven venoms—*Bothrops atrox*, *Bothrops jararaca*, *Bothrops nummifera*, *Crotalus adamanteus*, *Crotalus terrificus terrificus*, *Crotalus terrificus basilicus* and one of three specimens of *Crotalus horridus* were found therefore to contain a substance which, like thrombin or papain, reacted with fibrinogen to form a fibrillar gel indistinguishable from fibrin.

The clotting action of the above snake venoms was found to be independent of (a) the presence of calcium, shown by the fact that it occurred just as promptly in fibrinogen solutions containing sodium citrate; (b) the presence of tissue or platelet derivatives, shown by its occurrence in fibrinogen solutions which contained these factors only in minimal concentration. The velocity of the reaction was not affected at all by the addition of cephalin or tissue extracts to the fibrinogen; (c) the presence of prothrombin, for the prothrombin-free fibrinogen which was unaffected by Ca+cephalin, was nevertheless clotted by the venoms. These facts showed that the formation of fibrin was catalyzed directly by the snake venom. The eleven venoms which did not clot fibrinogen were found to hydrolyze protein and render it indifferent to thrombin.

The venoms arranged in order of their gelatin-splitting activity showed also proportional clot-forming activity. When the proteolytic activity of certain venoms was found to be below a certain level they also failed to manifest any effect on fibrinogen. On the other hand, some of the venoms which manifested a high degree of proteolytic activity rendered the fibrinogen indifferent even to thrombin.

c. **The Activation of Prothrombin to Thrombin by Snake Venoms.** Three of the 17 venoms—*Notechis sentatus*, *Bothrops atrox*, *Bothrops jararaca*—used in dilutions as high as 1:1,000,000 (the first two named in 1:10,000,000 dilutions), regularly catalyzed the transformation of prothrombin to thrombin. The mixed venoms of *Micrurus* and *Crotalus terrificus basilicus* were found to be weakly active. *Notechis scutatus* and a mixed *Micrurus* venom did not show any effect on fibrinogen; their activity on plasma noted previously was ascribed to their property of transforming prothrombin to thrombin. The other three venoms activated prothrombin and acted also on fibrinogen.

Despite the fact that these venoms represent a heterogeneous mixture of substances, the first 3 were found to be many times as effective

in this respect as crystalline trypsin. A 1:2,000,000 dilution of *Bothrops atrox* often produced a complete activation of prothrombin to thrombin; and 1:25,000,000 dilution manifested a definite, if partial effect. The reacting proportions therefore varied from 1:1000 and 1:10,000, which can be interpreted as evidence of a catalytic process and different from simple chemical combination.

As with trypsin, the transformation of prothrombin with snake venoms was independent of calcium ion, platelets or tissue derivative (cephalin). Moreover the rate of transformation and the amount of thrombin formed were not affected by the addition of these factors.

From the above discussion it is apparent that the clot-forming agents in snake venoms act in a manner comparable to enzymes. The question as to what degree these enzymes contribute to the pathological symptomology arising from snake poisoning and what is the direct bearing of the neutralizing property of antivenom immune sera on the anti-enzyme immunity will be taken up next.

**d. Anti-Thrombin, Anti-Proteolytic, and Anti-Necrotic Activities of Antivenin.** In a meeting of the British Medical Society, Stephens and Myers (1898) discussed a demonstration held in 1896 in the Physiological Society by Professor Kanthack showing that cobra venom mixed with shed blood in a test tube prevented fibrin formation; he also showed that this action could be prevented by previously mixing the poison with antivenomous serum—the mixture clotting as normal blood did; and finally he showed that the action of the serum was specific. This appears to be an early observation on the proteolytic activity of snake venom and its neutralization by antivenomous serum. According to Vellard (1930) the neurotoxic, clot-forming, and proteolytic properties of snake venoms are the most important from the point of view of toxicity. *In vivo*, while the clot-forming activity of venom causes more or less extensive intravascular thrombosis, the proteolytic activity of venoms may render the serum difficult to clot, maintaining the blood in the vessels in fluid form for a long time even after death has occurred. Autopsies of the animals confirmed the *in vitro* studies. The local necrotic action of certain venoms is due principally to their clot-forming and proteolytic properties; one can study this effect satisfactorily *in vivo* by intra-dermal injection in the ear of a rabbit of a sublethal dose of venom. *In vitro* experiments show



that antivenin neutralizes the clot-forming, proteolytic and necrotic activities of venom.

Discussing the relation of antitoxic, anti-thrombic and antiproteolytic properties of a given immune serum he found that these three properties do not appear simultaneously. Anti-thrombin appears first, then anti-necrotin (antitoxin) and last anti-proteolytic antibody. During the first month of immunization there is a great difference between the anti-thrombin and antitoxin. One ml. of serum of one of the horses immunized for three months with the venom of *C. terrificus* neutralizes *in vitro* the clot-forming activity of 0.7 mg. of this venom, whereas it neutralized the toxin activity of only 0.15 mg. of venom. The same anti-thrombin titer was maintained throughout this period while the antitoxin property increased progressively, attaining the power after six to seven months of neutralizing with 1 ml. from 0.5 to 0.7 mg. of venom. In the serum of animals immunized for a long time with *C. terrificus* the relation between anti-thrombin and antitoxin is sufficiently constant and the titer of the former is an approximate indicator of the latter, which is in contrast to their relation at the beginning of immunization. In the titration of antivenom sera the neutralization of the neurotoxic effect of *C. terrificus* is the main property. In the case of the venom of *Lachesis* the clot-forming action plays the important rôle. Vellard found that the specificity of antivenom sera does not obey a general rule. The study of various samples of antivenins showed that some of them exercise more anti-thrombin, and the other more anti-neurotoxic activity.\*

In a similar study on the enzymic activities of snake venoms Githens (1941; also Githens and Wolff, 1939) made the following observations. The venoms of crotalidic snakes injected subcutaneously or intradermally induced a local reaction characterized by necrosis of tissue, hyperemia and edema, the last being seen especially with subcutaneous injections. The reaction was studied on guinea pigs injected subcutaneously over the hip, or intradermally on the flank. A 1:1000 solution of venom was found most suitable for this study; more dilute solutions engendered a more extensive edema but with less necrosis. The skin near the site of injection soon showed a yellowish, or more commonly a reddish-brown discoloration, which reached its maximal extent in about two days, and was surrounded by

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\*For a review on animal venoms, see Essex (1945).



an area of hyperemia and edema. These local reactions are common to crotalidic venoms and the evolution of the lesion is essentially the same with all the venoms studied.

The neutralizing effect of antivenom serum was tested by mixing a fixed volume of antivenom serum with varying doses of venom and injecting after a reaction period of two hours at room temperature. The local effect of the venom developed so rapidly that no beneficial result could be obtained from antivenin given after the venom had been injected. In a subcutaneous test, 0.5 ml. of crotalidic immune globulin prevented the necrotic action of each of the venoms in doses of 1 to 2 mg. The neutralizing effect of antivenin was also marked in the intradermal tests. As the result of comparative tests Githens found that quantitatively, the neutralizing power of antivenin is equal to, or greater than, that on the neurotoxic factor of the same venoms.

The mechanism of the clotting of blood plasma *in vitro* has already been discussed in the preceding pages. Eagle and others had shown that some venoms act as thrombin causing clotting of solutions of fibrinogen, while others merely activate prothrombin. The failure of certain lots of snake venoms was interpreted as due to either the absence of the clotting factor or to the preponderant action of proteolytic constituents. In the latter case, it was stated that clotting may result with small amounts of venom, but fail to appear with larger amounts.

In a typical clot-forming test with fer-de-lance venom, Githens reported the following results on the clotting of 1 ml. of horse blood plasma.

(a) 0.001 mg. of venom caused the beginning of a clot in 8 minutes; the clot was soft in fourteen, and firm in 30 minutes.

(b) 0.000,03 mg. of venom caused the beginning of a clot in 10 minutes; the clot was soft in 35 minutes, and did not become firm.

(c) 0.000,01 mg. of venom caused the beginning of a clot in 60 minutes; did not become soft.

The time required for the formation of soft clot being more constant was selected as a standard. The minimal effective dose was defined as that which induced formation of a soft clot within 30 minutes. Fifty lots of venom were tested, representing 26 species of pit viper, two species of true viper and one elapine snake. Of these, seven caused no clotting in any concentration. Seven others induced clotting irregularly with 0.01 mg. per ml. of plasma, but never with higher or lower

concentrations. Three venoms caused clotting uncertainly over a wide range of dose, but showed no consistent relation between concentration and clotting action. All other venoms showed a definite relationship between the concentration and the speed and degree of clotting. The venom of the fer-de-lance proved much richer in the clotting factor than any other. One part in 100 million parts (1:100,000,000) of plasma caused clotting.

In testing the neutralizing effect of antivenin on the plasma clotting property of venoms, 0.1 ml. of antivenin was added to the plasma and was thoroughly mixed before adding the venom. In general, Githens found the antivenins were slightly less effective against the clotting factor than against the neurotoxic and necrotic factors of the same venoms. In most tests 0.1 ml. of antivenin prevented clotting with no more than 0.03 to 0.1 mg. of venom.

With regard to the specificity of antivenin action the following is quoted from Githens' study: "In our tests, specificity is seen in better neutralization of most of the rattlesnake venoms by crotalidic and cascabel antivenins, while bothropic antivenin was most effective against bothropic venoms. On the other hand, the cascabel antivenin was not especially effective toward its specific venom."

In studies on the antiproteolytic property of antivenins Githens found that 0.3 ml. of antivenin prevented the proteolytic effect of as much as 1 mg. of some snake venoms, and was entirely ineffective and irregular with others. Both crotalidic and cascabel anti-venins showed stronger action against the venoms of a true viper, the daboia, and of an elapine snake, and the Australian tiger snake, than against those of many pit vipers.

The question as to whether the irregularity and the absence of a more striking antiproteolytic neutralizing property of antivenins, as shown in this study, is due to the absence of more suitable experimental procedure, or to the weak antigenicity of the proteolytic enzymes of venoms must be left open for the time being.

The above experimental data show that the toxicity of snake venoms is related to the activity of various enzymes all of which are neutralized by antivenom immune sera. The physiological consequences of the hemolytic enzyme, lecithinase, of snake venoms, and its neutralization by antivenom sera will be discussed in a succeeding section of this book.

## 5. Staphylococcal and Other Bacterial Factors in Fibrin Clot Formation

It has been known for several decades that certain staphylococcal strains produce a factor which promotes the formation of fibrin clot. During the last decade several reports have shown that other bacterial species elaborate a similar factor. This factor has been called "*Coagulase*." For reasons stated at the beginning of Section C, and also in view of the inadequacy of available information concerning its chemical nature and its role in fibrin clot formation, the term coagulase seems to be a misnomer. We prefer to call it, at present, a *Clotting Factor* to be used exclusively in discussions pertaining to plasma clotting.

**a. Distribution of Plasma Clotting Factor among Bacteria.** *Staphylococcus aureus* (*pyogenes*) has principally been studied as a good producer of clotting factor. *Staphylococcus albus* has been reported variously to possess or lack this ability. Reed, *et al.* (1943) studied several gas gangrene species with respect to their ability to clot plasma. Some 25 cultures belonging to the six most important gas gangrene species: *Cl. welchii*, *Cl. septicum*, *Cl. novyi*, *Cl. sordellii*, *Cl. sporogenes* and *Cl. histolyticum* were tested for ability to clot guinea pig plasma by the methods ordinarily employed with staphylococcus cultures. Two of five cultures of *Cl. novyi* and one of five cultures of *Cl. septicum* regularly produced rapid clotting; *Cl. histolyticum* and *Cl. sporogenes* produced slow clotting. Other cultures failed to produce clotting. On the basis of these results, these investigators concluded that plasma clotting is not a significant characteristic of this group of species. However, the experiments of Reed, *et al.* may merit reinvestigation by taking into consideration several factors not considered during their experimentation, since experiences of other workers, and particularly those of Smith and Hale (1944), to be discussed below, indicate that guinea pig plasma is unsatisfactory when used in testing for the presence of clotting factor.

**b. Anti-clotting Factor.** Several investigators have reported the production of another factor by bacteria which interferes with or inhibits blood clotting. Dennis and Berberian (1934) discussed the relation of this factor to inflammatory fixation of various strains of streptococci

and *Staphylococcus aureus*. Witebsky and Neter (1936), Neter and Witebsky (1936), and Neter (1937) reported that anti-clotting factor was produced by *Streptococcus viridans*, enterococci, pneumococci of various types, some strains of *Escherichia coli*, *Pseudomonas pyocyaneus* and others. Reed, *et al.* (1943) reported that six cultures of *Cl. welchii* out of thirty-three tested prevented calcium chloride from clotting rabbit plasma. About the same proportion of cultures of *Cl. novyi*, *Cl. septicum*, *Cl. sporogenes* and *Cl. histolyticum* exhibited an anti-clotting effect. The anti-clotting factor was reported by Reed, *et al.* to be less active against guinea pig or human plasma than against rabbit plasma.

According to Dart (1936) streptococcal anti-clotting factor is not specific for human fibrin, but will also prevent the clotting of isolated-fibrinogen-thrombin complex from rabbit, sheep, cow and domestic swines. The anti-clotting factor is not neutralized with concentrations of commercial streptococcal antiserum sufficient to neutralize streptococcal fibrinolytic factor. The fibrinolytic factor is precipitable with alcohol, the anti-clotting factor remains in solution in the supernatant alcohol. The anti-clotting factor recovered from alcohol is thermostable, resisting heating at 100°C. for 30 minutes.

There is lacking information concerning the specific nature and action of the anti-clotting factor; also the extent of the distribution of bacterial clotting factor remains undefined.

**c. The Role of Bacterial Clotting Factor in Phagocytosis and Infection.** In discussing the pathogenicity of a bacterium one must not lose sight of various factors they elaborate in the elucidation of the mechanisms of bacterial invasiveness and pathogenicity. Metabolism of a bacterium producing toxins etc. in a host environment no doubt plays a significant role. There are, however, accessory factors which may be essential, though themselves non-toxic, for the initiation and spread of an infection in a susceptible host. According to more recent studies (Hale and Smith, 1945; Smith, Hale and Smith, 1947) the staphylococcal plasma clotting factor is such an accessory factor in the fixation of staphylococci and the production of circumscribed local lesions. These investigators reported that clotting factor inhibits phagocytosis. Thus, during an infection plasma clotting activity confers upon the infective organism a first line of defense by resisting the phagocytic activity of leucocytes. Under these conditions, the organism



is able to elaborate its toxic products. It has been observed that the plasma of a species of host (horse, rabbit and human) which is susceptible to infection is clotted by the action of staphylococcal factor; and the plasma of those species (guinea pig, and mouse) which are resistant to infection do not respond to the clotting effect of staphylococcal factor. In the latter case, the animals have been reported to tolerate enormous doses of the same staphylococci, suggesting that in the absence of clottable menstruum the bacteria are easily phagocytized by leucocytes.

Inhibition of the phagocytosis of staphylococci by leucocytes is conceived as due to the formation of a fibrin envelope around the cocci. The clotting of plasma in an environment containing cocci and leucocytes produces compact masses, embedded in a fibrin matrix, forming a mechanical obstacle to phagocytosis quite apart from the specific mechanism of inhibition. Under these conditions leucocytes are entangled and immobilized and are prevented from ingesting the cocci which are clumped in the fibrin. Localization of a large number of leucocytes in this manner produces abundant pus formation.

The conversion of fibrinogen into fibrin on the surface of cocci producing sticky surfaces and thereby the agglutination of cocci has been demonstrated by the following observations. Birch-Hirschfeld (1934) (cited by Cadness-Graves, *et al.* 1943) observed that thick suspensions of staphylococci were rapidly clumped in human plasma. Cadness-Graves, *et al.* made use of this observation and developed a slide-test for rapid presumptive identification of potentially pathogenic *Staphylococcus aureus* (*pyogenes*), or those staphylococcal strains which produced clotting factor. Berger (1943) reported that all clotting factor-producing staphylococci were agglutinated by solutions of fibrinogen. Those cocci which failed to produce this factor were not affected. The clumping of pathogenic staphylococci in plasma is not a true agglutination and no immune body is involved. He suggested that the reaction is due to the ability of the cocci to form fibrin from fibrinogen on their surfaces and that this causes them to stick together. According to Smith, Hale and Smith (1947) the inhibition of phagocytosis due to the protective barrier of fibrin formed around the cocci as a result of clotting activity is a most important factor in the initiation and establishment of infection.

Smith and Hale (1944) reported that the non-clotting of the plasma



of some species and of occasional human beings in routine fibrin clot tests with positive staphylococci is due to a deficiency of an activator which is present in testicular extract and fresh human and rabbit serum. When this activator was added to a non-clotting system it readily formed clot. The effect of the addition of activator was also observed by promoting the inhibition of phagocytosis in a system containing plasma of certain species which failed to clot when the activator substance was not added. Smith, *et al.* (1947) reported that the intradermal inoculation of a guinea pig with non-clotting staphylococcus results at most in a trivial reaction in spite of its  $\alpha$ -toxigenicity ( $\alpha$ -hemolysin). In contrast, guinea pigs treated with human plasma, containing the activator, showed striking results. In every case a large edematous, inflammatory swelling developed, followed by extensive abscess formation and necrosis. With the larger doses of the staphylococcus, ulceration ensued with the free discharge of pus, and the spread of infection along the flanks and to the mid-abdominal line necessitated early sacrifice of the animal. This same dose of human plasma, given without staphylococci, was without apparent effect apart from the transient swelling produced by the large inoculum.

Smith, *et al.* (1947) were cognizant of the fact that the extensive cellular necrosis of the characteristic focal lesions is evidence of toxic action in which clotting factor can have no direct part; these indications were considered to be due to the production of  $\alpha$ -toxin, leucocidin and possibly other unknown tissue toxins. Toxin, however, cannot be elaborated until the organism has gained a foothold in the host and begun to multiply, and they suggested that at the initial stage of natural infection with a small number of staphylococci, coagulase production is absolutely essential if the infection is to progress. Subsequently, the clotting factor may aid the further multiplication of the organism in defiance of the ensuing phagocytic response, and may determine the outcome when infected emboli are transported to other sites.

**d. Comment on the Nature of the Staphylococcal Clotting Factor.** The ability of *Staphylococcus aureus* (*pyogenes*) to clot blood or plasma was observed several decades ago. Despite a considerable number of studies the chemical nature and the mechanism of its action have, as yet, remained obscure. Recently, Smith and Hale (1944) reported the results of a fairly comprehensive study. The conclusions

they arrived at, however, appear to be open to question. They reported that coagulase by itself is inert as far as the conversion of fibrinogen to fibrin is concerned. It is, however, presumed to be the precursor of a thrombin-like substance which can convert the fibrinogen of all species, so-far tested, into fibrin clot. The formation of thrombin-like substance requires the participation of an activator; this is present in adequate quantity in some plasmas. The reaction is therefore considered to be analogous to normal thrombin formation from prothrombin by the agency of thrombokinase, with the important difference that calcium is not required. The experimental data reported by them and other investigators do not support these conclusions and pattern of thought. The data as discussed below, may permit, however, the formulation of a different mechanism which could account for the experimental facts.

e. "Activation" of the Staphylococcal Clotting Factor. Smith and Hale (1944) observed that a testicular aqueous extract is a rich source of the activator substance which added to non-clotting plasma of certain species produces fibrin clot promptly. Human and rabbit plasma respond to clotting (without the added activator), and guinea pig plasma fails to clot under the same conditions. They reported that guinea pig plasma clots at 20°C. but not at 37°C., though clotting at the higher temperature was slow. On the other hand, mouse and fowl plasmas failed to clot at any temperature. The addition of either human or rabbit testis extract to guinea pig, mouse or fowl plasmas was found to render them as fully susceptible to the effect of staphylococcal factor as were human and rabbit plasmas. Human and rabbit sera were also found to serve as sources of activator substance. The failure of occasional samples of human plasma to clot with staphylococcal strains known to produce clotting factor was attributed by experimental verification to a deficiency of activator substance. The addition of testis aqueous extract caused atypical plasma to behave like the normal in every respect.

They reported that clotting factor alone is entirely without effect on purified fibrinogen-prothrombin because of the removal of all activator substance. This fibrinogen readily clotted on the addition of clotting factor and testis extract. The combination of clotting factor and testis extract was considered to serve as thrombokinase (thromboplastin). The clotting of this fibrinogen by activated clotting factor was

more rapid than the clotting of plasma; even so the action of thrombokinase in the presence of calcium chloride was still more rapid. In the absence of information concerning the quantities of various factors used in these systems the differences in the rates of reactions do not appear to lend much aid in the interpretation of observed effects.

In connection with the above postulates the following observations are of interest. Miale (1949) observed that sterile cell-free staphylococcal clotting factor ("coagulase") or whole culture of staphylococci fail to cause the clotting of pure fibrinogen, but would clot oxalated plasma during a period of from 15 minutes to several hours. This indicates that a reaction must take place between a plasma component (coagulase-globulin) and the staphylococcal factor before fibrinogen can be converted to fibrin. Progressive removal of calcium in no way interferes with this clotting of plasma by either staphylococcal factor or when it is in "combination" with the plasma component, even when the plasma has been rendered non-clottable by an excess of thromboplastin. It would mean that the effect of staphylococcal factor differs basically from the activation of prothrombin to thrombin, calcium ion being essential for the latter reaction. This conclusion was supported by the failure of heparin, azo dyes (chlorazol fast pink, Nat. Aniline, C. I. №353), and soluble fluorides (decalcifying agent) to interfere with the clotting in the presence of staphylococcal factor and plasma globulin co-factor, since heparin is known to inhibit thrombin activity and the activation of prothrombin, and the azo dyes are assumed to inhibit, *in vivo* and *in vitro*, thromboplastin.

**f. Properties of Clotting Factor and Activator Substance and Their Possible Role in Fibrin Clot Formation.** The above cited interpretations offered by Smith and Hale are difficult to accept and appear to be contradictable by the following considerations. The staphylococcal clotting factor is heat-stable, its antigenicity has been disputed and claimed\* and therefore appears to be a substance of unknown nature. As such it does not appear to warrant the assumption that it is a

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\*Tager and Hale (1948) suggested that staphylococcal clotting factor is antigenic for some rabbits. Three of the nine rabbits which received a long course of inoculations with a highly purified clotting-factor possessed sera which retarded considerably the clotting time, showed complement fixation titer, and colloidal agglutinating property. For the demonstration of these properties in these antisera, the presence of alpha hemolysin in the inoculated material was a necessary condition. These findings do not, however, interfere with the discussion which will follow.

potential enzyme. The assumption, therefore, that the activator substance converts the clotting factor to a thrombin-like substance, or that the clotting factor is a precursor of thrombin-like substance is negated by the above mentioned properties of the clotting factor, for thrombin-like substance must necessarily be of protein nature and relatively heat-labile. The clotting factor has not been shown to exhibit any of these properties.

The thermostability of the clotting factor appears to compare with the heat stability of thromboplastins. Confirming the findings of previous investigators, Smith and Hale (1944) reported that staphylococcal culture filtrates possessing strong clotting activity retained considerable activity after heating for thirty minutes at 100°C. but with a steady progressive loss of activity with time. Heating at 80°C. causes some loss, complete inactivation is rapid at 120°C. No mention was made of the use of an experimental precaution to exclude air-oxygen during heating as a possible cause of oxidative inactivation of lipid-like substances.

Quick (1942) reported that thromboplastin when exposed to air gradually turns brown and simultaneously with this a loss of activity occurs. Exclusion of air protects it indefinitely, presumably by preventing oxidation. Quick determined the effect of heating on the thromboplastic activity of rabbit brain and lung extracts. Heated at 54°C. for 15 minutes the clotting times for brain and lung extracts were, respectively, 8 and 7.5 seconds; at 75°C., 25 and 12 seconds. Heated for 10 minutes at 100°C., they were, respectively, 37 and 15 seconds, and heated for 60 minutes at 100°C. they were, respectively, 50 and 20 seconds. These results suggest a comparability between thromboplastin and the clotting factor with respect to thermostability. A final understanding of the significance of this relationship requires critical analysis of the chemical nature of the clotting factor.\*

\*Walker, *et al.* (1947) reported that "coagulase" contains peptide linkages hydrolyzable by proteolytic enzyme preparations. However, in view of the fact that "coagulase" resisted the action of 120° C. temperature for 20 minutes in the autoclave they raised some doubt about its enzyme nature. They used 20 mg. of commercial trypsin (Pfaffstiehl) and U.S.P. pepsin (Merck) to digest five ml. of the culture supernatant as source of "coagulase." Both the U.S. Dispensary (1943) and U.S.P. (1947) state that commercial proteolytic enzyme (pancreatin) is a substance containing enzymes, principally amylase, trypsin, and lipase (steapsin). Since they used 20 mg. of commercial enzyme all of these enzymes can be assumed to be present. In view of the result of our analysis of the available data, indicating that "coagulase" may be a lipid type of compound, and in view of the uncertainty of the antigenicity of "coagulase,"



The substance in testicular extract, assumed to be the activator of the clotting factor, is reported to be a protein, non-dialyzable, and, possibly, antigenic. If we make an attempt to harmonize these properties of the activator substance and the clotting factor with the plasma clotting reactions described above we may, perhaps, be permitted to suggest the following interpretation as the probable course of the reactions.

The staphylococcal clotting factor may represent a complex consisting of lipid-like substance and X-component. The X-component blocks the activity of the lipid-like substance, keeping it in an inactive state. The X-component manifests a greater affinity for a certain serum component and testicular material (used as activators), forming a non-dissociating or weakly dissociating complex. Under these conditions the lipid-like component of the staphylococcal factor would be set free to convert prothrombin to thrombin, the latter converts fibrinogen to fibrin clot. These suggestions may perhaps be schematized in the following manner:

Clotting factor  $\rightleftharpoons$  Lipid-like active substance + X-component

Serum  
 or  
 testicular  
 ↓  
 protein(?)

Lipid-like active substance + serum or testicular protein-X complex

Prothrombin + staphylococcal lipid-like substance  $\rightarrow$  thrombin

Plasma fibrinogen + thrombin  $\rightarrow$  fibrin clot

The degree of affinity of the X-component for the species serum or testicular proteins would appear to determine the amount of uncombined active component of the staphylococcal clotting factor at a given temperature. The non-clotting behavior of guinea pig plasma at 37°C. and its clotting at 20°C. for example, would point to a greater dissociation of X-plasma protein complex at 37°C. than at 20°C.:\*

37°C.

X-Protein complex  $\rightleftharpoons$  Protein (of guinea pig or mouse plasma) + X  
 20°C.

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the action of commercial enzyme preparation may involve the hydrolysis of a lipid-like or polysaccharide-like substance, and not a protein.

\*In connection with the temperature effect on serological reactions the following observations are of interest. Filitti-Wurmser and Jacquot-Armand (1947a, 1947b) de-



At 37°C. the testicular protein appear to exercise a greater affinity for the X-component than the latter exercises for the active lipid-like substance of staphylococcal material. Under these conditions the testicular protein would give the appearance of exercising the role of an activator by combining with the staphylococcal X-component and setting free the lipid-like active substance to convert prothrombin to thrombin.

Differences in the degree of clotting of various plasmas can likewise be explained by differences in the affinities of species serum proteins for the staphylococcal X-component. This is not an unusual behavior, for it has been shown that cat, horse, human and rabbit etc. sera show different degrees of affinities for a given substance. There is also sharp demarcation among the affinities exercised for a variety of given substances by various components of the serum of a species. Within a given bacterial cell there reside various enzyme proteins with varying or absence of affinities for a chemotherapeutic drug (see further Sevag, 1946).

In summary it would appear that the above considerations at present do not lend support to the postulated enzyme nature of the staphylococcal clotting complex. The data may suggest that it is a lipid-complex.

## 6. Antibody Against Rennins

**a. Certain Properties of Rennin.** Rennin, found in the gastric juice of the fourth stomach of the calf, catalyzes the clotting of milk. Pepsin, at faint acidities, and chymotrypsin at neutrality also clot milk. Rennin differentiates itself from pepsin by its stability at pH 9.0. At this pH

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terminated the degree of dissociation of hemagglutinated systems at 37°C. 25°C. 15°C. and 5°C. They found that the rate of agglutination between the specific serum and red blood cells increases as the temperature at which the reaction takes place is lowered. In going from 37°C to 5°C the equilibrium is displaced and the degree of agglutination at 37°C was found to be about one-third of that at 5°C.

The results of a study on the energy relationships of the agglutination reaction involving red blood cells by Filitti-Wurmser and Jacquot-Armand (1947c) show that this is a reversible reaction and obeys laws of mass action. The equilibrium between blood corpuscles and the agglutinins varies with the temperature in the direction corresponding to an exothermic reaction. A calculation by Filitti-Wurmser, Jacquot-Armand and Wurmser (1948) of the ratio of the equilibrium constant  $K$  at 25°C. to  $K$  at 37°C.  $K_{25}/K_{37}$ , yielded a value of 3.5 which is said to correspond to an enthalpy of -19000 calories. Assuming that the combination between a molecule of agglutinin and a blood corpuscle is through hydrogen bonds, this energy is said to correspond to three to four of such bondings per molecule of agglutinin.

pepsin is inactivated very rapidly (Sumner and Somers, 1947). Rennin is inactivated by strong acids. Pepsin is most active at pH 1.5 to 2.0.

One part of rennin has been reported to clot 4.5 million parts of milk at pH 6.2 and 37°C. (Tauber and Kleiner, 1932, 1934), and 72 million parts of fresh raw skimmed milk at pH 5.8 and 40°C. in 10 minutes (Hankinson and Palmer, 1942).

Rennin has been isolated in the form of needle shaped crystals (Hankinson, 1942, 1943), and in the form of flat plates (Berridge, 1943).

**b. Mechanism of Milk Clotting.** According to some investigators, rennin acting on casein splits it yielding acidic and basic groups in the molecule. Calcium ion in the milk combines with these groups producing a gel of calcium-phosphocasein (casein contains 0.85 per cent phosphorus). The resultant gel is a polymer of casein or paracasein (see Nord and Weidenhager, 1940). According to another interpretation rennin splits casein through hydrolytic cleavage into soluble paracasein and peptone-like products. Calcium ion in the milk combines with paracasein forming the insoluble calcium-paracasein complex. The question of which of these, or any other, interpretations correspond to the true mechanism of milk clotting cannot as yet be definitely stated.

**c. Milk and Plasma Clots Compared.** The clotting of milk by rennin and that of plasma by thrombin yield products which, in certain respects, seem to lend to comparison. These are:

- (1) Formation of insoluble elastic products from soluble protein substrates. At certain moderate temperatures milk clot yields strings with increasing elasticity.
- (2) Calcium ion is needed in both clotting processes. In neither case does clotting occur following pretreatment with oxalate or citrate ions which bind calcium ion.
- (3) Both clots show syneresis. In plasma clot, non-fibrinogen plasma components, and in milk clot, a clear fluid called whey, separate out.

**d. Antibody Against Animal Rennin.** In order to find a relationship between toxin-antitoxin and enzyme-antienzyme reactions Morgenroth (1899) undertook the following investigation in Ehrlich's laboratory. He immunized goats with rennin and the immune serum was shown to inhibit the milk clotting activity of rennin. Since

the controversy regarding the existence of anti-rennin antibody will be analyzed below, we will begin with the description of Morgenroth's results.

*Immunization of Goats with Rennin.* The rennin preparation used for the subcutaneous immunization of goats was prepared as follows: A 10 per cent sodium chloride suspension of commercial Witte rennin was shaken mechanically for a prolonged period. After centrifuging, the insoluble sediment was discarded. The clear supernatant was diluted with sterile water for injection. By a gradual increase of the dose of rennin as much as 6.5 g. were injected subcutaneously into each of two goats. One of the goats yielded a highly potent immune serum; the other, not as good. A permanent stock-enzyme solution was prepared which showed no decrease in activity during 18 months.

After numerous preliminary experiments with respect to variations of time, temperature and concentration, he developed a reliable method for testing the clotting of milk with the enzyme solution. By allowing the mixture of rennin and milk to react overnight at a temperature of 0° to 8°C. before taking readings, consistent and reliable results were obtained. His rennin solutions were carefully neutralized for daily use to neutral red (pH 6.0) with a lactic acid solution. The activity of the rennin solution in clotting cow's milk (about pH 6.58) was (by dry weight) 1 part in 3,000,000.

The anti-rennin potency of the immune goat serum was determined as follows: The cow's milk was treated with a volume of immune serum to have a 2 per cent serum concentration in the mixture. To each 5 ml. portion of this mixture increasing amounts of rennin solution were added at various intervals and parallel control tests were carried out in the absence of immune serum to determine the relative time of the clotting of the milk. In contrast to the control value of 1:3,000,000, in the immune reaction mixture containing 1 part of rennin in 30,000 there was no clotting of the milk. When the concentration of rennin reached 1:25,000 clotting was observed. These facts showed that a 2 per cent immune serum neutralized more than 100 times the amount of rennin present in the control test. Another immune serum tested identically was shown to neutralize 200 times the amount of rennin present in the control reaction system.

Morgenroth found that 0.05 ml. of diluted rennin solution was capable of coagulating 30 liters of milk. Thirty ml. of immune serum was

required to neutralize the activity of rennin contained in 0.05 ml. solution, and 0.025 ml. and 0.015 ml. of rennin solution were respectively neutralized by 15 ml. and 10 ml. of immune serum. In control experiments normal goat serum showed no effect on the coagulation of milk by rennin.

While the milk of cows and normal goats was coagulated with as little as 1 part in 3,000,000, the milk of the goat immunized with rennin did not coagulate until the concentration of added rennin was 1 part in 30,000; that is, 100 times more rennin was required to coagulate the milk of an immune goat than that of a normal goat. This was regarded as a confirmation of the facts previously observed by Ehrlich that the milk of immunized animals contains antitoxins, such as tetanus and diphtheria antitoxins. And the amounts of antitoxins and antirennin were found to run parallel with the degree of immunization.

**e. Antibody Against Plant Rennin.** In a subsequent study Morgenroth (1900) experimented with a plant rennin prepared from an Italian plant, *Cynara cardunculus*, used for the preparation of cheese. He obtained a partially purified enzyme preparation twenty times weaker in activity than that of the animal rennin, and with this immunized a goat. Using 5 ml. of cow's milk, the immune goat serum prepared against plant rennin neutralized 27 to 30 times more rennin than was required by the control tube. The anti-rennin (plant) serum did not neutralize the activity of animal rennin. Conversely the immune goat serum against animal rennin did not neutralize the activity of the plant rennin. These facts showed the serological specificity of the animal and plant rennins.

Thaysen (1915) prepared potent immune sera by injecting rennin into rabbits. Those sera could be preserved for over a year without observing any decrease in their specific activity. Tests were performed similar to those used by Morgenroth. In inhibition experiments the activity of 0.03 ml. of rennin solution was completely neutralized instantaneously by 0.1 ml. of homologous immune rabbit serum at 20°C. Normal serum showed no inhibitory action on rennin, or, if any, the effect was not observed for four hours or longer. The instantaneous action of the immune serum on rennin activity, as observed by Thaysen, agrees perfectly with the facts known at present. In serological reactions 90 to 95 per cent of an antigen-antibody combination occurs within the first few minutes.



Thaysen did not believe that the neutralizing power of the immune serum was due to a specific antigen-antibody reaction. He postulated two possibilities: (a) a pH difference between immune and normal sera; (b) an increase in the concentration of "adsorbing bodies" or "some substance" in the serum of immunized rabbits, which apparently does not exist in the sera of normal rabbits. Since he did not offer any experimental data regarding his "adsorbing bodies," as being different from antibodies, this idea must be dismissed as speculation. An analysis of Thaysen's data will also show that his other postulate lacks any experimental basis; 0.2 ml. of immune serum (ca. pH 7.8) is incapable of alkalinizing and thereby inactivating 1 ml. of rennin solution (the optimal activity lies in the range of pH 6.0-6.4) in 10 ml. of milk (pH 6.58) which possesses very strong buffering capacity (Moser, 1927). If there ever exists any inactivation following the action of alkaline immune serum on rennin, this effect will be reversed on being exposed to the reactivating pH of the milk. This is illustrated by the following facts. Kleiner and Tauber (1932) made a calcium carbonate extract of the mucosa of the fourth stomach; the pH of the extract was 7.4. One ml. of this extract clotted 30 ml. of milk in 10 minutes at 40°C. without the addition of acid to the extract. The rennet activity of this extract was lost entirely only after two-day incubation at 37°C. As soon as the pH was adjusted to 5.3, the immediate milk clotting activity was 1:333; activity after one day's incubation at 37°C. was 1:420. The reversible inactivation of rennet compares very favorably with those of other enzymes (Northrop, 1939).

## H. ENZYMATIC AND PHARMACOLOGICAL ACTIVITIES OF HEMOLYTIC SUBSTANCES

### 1. Hemolytic Lysolecithin Derived by the Action of Snake Venom Lecithinase

Stephens and Myers (1898) reported that on mixing cobra venom with guinea pig blood *in vitro* they observed hemolysis and the delay or complete absence of clotting of blood. Investigating this observation further, they found that the hemolytic action of 0.1 mg. of venom on guinea pig blood was completely arrested by 0.1 ml. of immune serum. The reaction was specific, as other horse immune sera, such as diph-



theria antitoxin or tetanus antitoxin, possessed no such power. A guinea pig weighing from 250 to 350 g. succumbed in five to eight hours after receiving 0.1 mg. of cobra venom. The hemolytic action of this quantity of poison, as shown above, was neutralized by 0.1 ml. of immune serum, and such a neutral mixture was never found to be fatal to the animal, but if the hemolytic action was incompletely neutralized the animal might either survive or die. These findings suggested a direct relationship between the antihemolytic and the *in vivo* protective power of the immune serum; they found, however, that when higher amounts of venom were treated with the equivalent amount of antiserum the protection was not always obtainable, which they believed was due to the presence of other non-neutralized toxic factors in the venom.

a. **Discovery of Hemolytic Lecithinase and Its Neutralization by Antivenin.** Later studies showed that the hemolytic action of snake venom was due to the lipolytic action of venom lecithinase on the lecithin of red blood cells, resulting in the production of hemolytic lysolecithin and lysocephalin. The lecithinase activity was found to be completely neutralizable by antivenomous serum; that is, the enzymatic formation of hemolytic lysolecithin and lysocephalin was prevented by antivenom. In addition to the antinecrotic, anticlotting and antiproteolytic properties, the antivenom must also possess anti-lecithinase activity in order to account for its protective property *in vivo*. The discovery of lecithinase progressed as follows. Flexner and Noguchi (1902) at the University of Pennsylvania discovered that in no instance were *washed blood corpuscles* hemolyzed by venom. If the separated serum was restored to each of the several kinds of blood corpuscles treated with venom, lysis took place. This observation was the beginning of a chain of discoveries which established the fact that the hemolysis of red blood cells by snake venom was mediated by the enzyme lecithinase. Two years later Kyes (1903, 1904), of the University of Chicago, working in Ehrlich's laboratory, found that if cobra venom is brought into contact with lecithin a hemolytic substance is produced. He also showed that anti-cobra venom horse serum neutralizes the action of numerous snake venoms on lecithin, apparently preventing the formation of the hemolytic lecithin derivative.

Von Dungern and Coca (1908) reported that the hemolytic deriva-

tive of lecithin is nothing but a lecithin from which oleic acid is split by the hemolytic enzyme of the snake venom. They called this lecithin derivative *desoleolecithin*. In contrast to lecithin, this substance was found to be insoluble in ether and water, and represented 60 per cent of the lecithin used. The amount of oleic acid split off was calculated to be 30 per cent. They also found that the partially purified venom lecithinase and that present in cobra venom are completely neutralized by Calmette immune serum.

Manwaring (1910) investigated the lecithin hydrolyzing factor in cobra venom and reported that it was a powerful lipase, and that the hemolytic activity of "mono-fatty acid-lecithin" (desoleolecithin of Von Dungern and Coca) accounts for the total hemolytic action of lecithin-venom mixture.

Delezenne and Ledebt (1911, 1912) reported that the hemolytic substance produced from lecithin by the lipolytic action of snake venom is soluble in water and alcohol, and insoluble in ether. It resists boiling temperature and is not neutralized by antivenom, though antivenom neutralizes the snake venom enzyme which produces the hemolytic substance. The venom does not enter into chemical union with lecithin during the formation of the hemolytic substance. They observed that the hemolytic power of the venom-serum, using horse serum as source of lecithin, declines and is completely abolished after reaching a peak. This was explained as due to the advanced action of lecithinase on lecithin beyond the hemolytic stage. When they added a titrated amount of antivenom to the venom-lecithinase mixture at a time when the hemolytic power is at a peak, they could prevent the activity of lecithinase and thus prevent the further hydrolysis of hemolytic desoleolecithin. Contardi and Ercoli (1933) reported that snake venom contains two lecithinases, A and B. Lecithinase A splits one of the two fatty acids from lecithin producing the hemolytic lysolecithin.\* This enzyme is incapable of hydrolyzing lysolecithin further. Lecithinase B, on the other hand, is capable of splitting both of the fatty acids from lecithin, or the remaining fatty acid from hemolytic lysolecithin, abolishing its hemolytic property and yielding choline-glycerin-phosphoric ester. The activity of lecithinase B evidently accounts for

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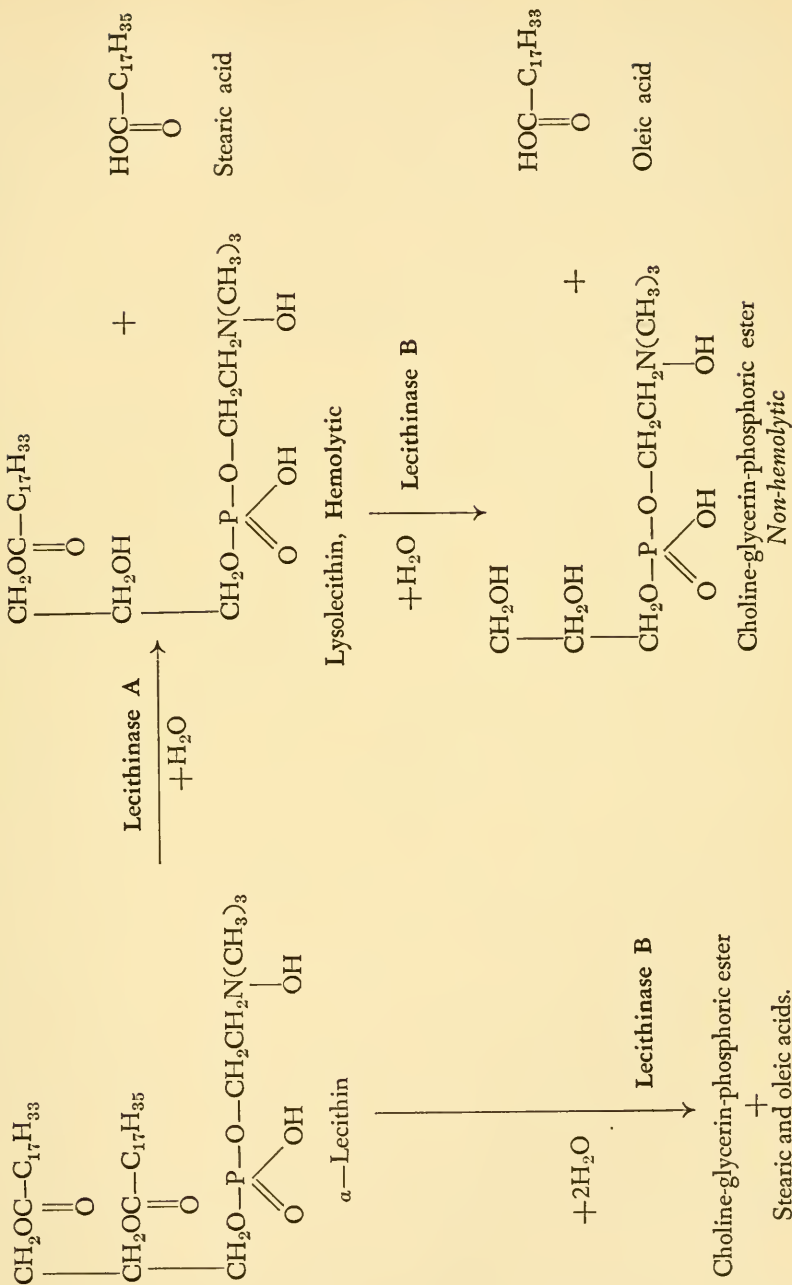
\*Gortner and Hermans (1943) reported that the number of erythrocytes of man, rabbit and sheep hemolyzed by 1 mg. of lysolecithin were, respectively,  $5.5 \times 10^9$ ,  $7.7 \pm 0.4 \times 10^9$  and  $15 \pm 2 \times 10^9$ .

the observation of Delezenne and Ledebt that the action of snake venom first progresses to the hemolytic peak and then declines. It is interesting to note that both of these enzymes were neutralized by antivenom serum as demonstrated by the findings of Delezenne and Ledebt.

In a subsequent study Delezenne and Fourneau (1914) undertook a systematic investigation regarding the nature of the hemolytic lecithin derivative. They found that fresh sterile cobra venom acting on vitellin from egg yolk at 50°C. transforms lecithin completely into a white substance having a high degree of hemolytic activity. One mg. of snake venom was found capable of transforming 200 g. of lecithin. The amount of lecithinase contained in 1 g. of crude snake venom was capable of catalyzing 200,000 g. of lecithin, which naturally excludes any possibility of a chemical reaction between venom and lecithin in a stoichiometrical sense. Furthermore the venom after its action on lecithin could be recovered and used again. They isolated from the reaction mixture the hemolytic substance in the form of rectangular rod-like crystals, and identified it as anhydrous monopalmitic lecithin (monopalmitophosphoglyceric ester of choline) which is now known as lysolecithin or lysocithin.

Levene, Rolf and Simms (1924) found, on the other hand, that the action of cobra venom on egg yolk yields a mixture of lysolecithin and lysocephalin. Twenty yolks were diluted with 600 ml. of M/15 phosphate solution of pH 7.0 (venom lecithinase is active at pH 6.5 to 7.5; it is entirely inactivated at pH 8). The phosphate suspension of egg yolk was digested with 0.1 g. of cobra venom for 14 hours at 40°C. A concentrate of the alcoholic extract was treated with a concentrated solution of cadmium chloride to precipitate lysolecithin and lysocephalin. Lysocephalin is the more insoluble in organic solvents and was purified by crystallization from a solution in chloroform. It crystallizes as transparent needles which soften at 140°C. and melt at 198°C. with decomposition. On hydrolysis of lysocephalin only one acid, namely stearic acid, could be isolated. Lysolecithin is very much more soluble than lysocephalin; it may be crystallized from chloroform, pyridine, and methyl and ethyl alcohols in aggregates of needles. It softens at 100°C. and decomposes at 263°C. (On hydrolysis lysolecithin yielded palmitic and stearic acids.)

Experiments carried out by Noguchi showed that both lysolecithin

*Lecithinases A and B of Snake Venom*

and lysocephalin were hemolytic; lysolecithin showed an activity three times as great as lysocephalin.

**b. Physiological Consequences of the Action of Lysolecithin.** The studies of Feldberg and Kellaway (1937) on perfused lungs of guinea pigs, cats, dogs and monkeys have shown that the injection of snake venoms produces severe changes in the lungs and causes the appearance of coagulable protein and of histamine in the outflowing fluids. Increasing doses of venom caused increasing loss of histamine and the amount of coagulable protein in the outflowing fluids also increased. Those samples of perfusate containing the highest histamine concentration were richest in protein.

In the perfused lungs the venoms caused broncho-constriction which was accounted for by the liberated histamine. The other changes produced, however, could not be imitated by histamine. *Crotalus atrox* caused the destruction of tissue. This effect, peculiar to this venom, was considered to be due to proteolysis. Three venoms were studied, all of which had one result in common—the swelling of the lung due to accumulation of fluid and the appearance of glossy patches. As these changes were paralleled by the loss of histamine from the lung, it looked as though they might be caused by histamine.

Following the above study with snake venom Feldberg and Kellaway (1938) conceived the idea that the formation of hemolytic lysolecithin by the venom lecithinase might account for effects of the venom other than hemolysis, and that its formation in the tissues might make the cells permeable to their histamine. Consequently they found that the active substance or substances responsible for the above symptomatology could be extracted with methyl alcohol from the venomated liver of the monkey; and the actions of such extracts were compared with that of "lysolecithin" obtained from an alcoholic extract of lecithin treated with cobra venom. Both extracts had similar effects on widely different tissues, but there were some quantitative differences. At least part of the hemolytic activity of both extracts was attributable to lysolecithin; and the quantitative agreement between the extracts in hemolytic power, in ability to liberate histamine, and in toxicity for the guinea pig suggested that all these actions result from the presence of lysolecithin. Since the active substances in both extracts appeared to be formed by the action of venom on lipids, provisionally their total activity was regarded as being due to lysolecithin-like substances. The



above experiments were repeated by Feldberg, Holden and Kellaway (1938) with a highly purified lysolecithin. On the whole, the same results were obtained as with the methyl alcoholic extract of the venom-treated lecithin.

In view of the important nature of these observations and their possible similarity to the action of bacterial toxins, we quote here the conclusions of the above studies as given by Feldberg and Kellaway:

*"Conclusions.* 1. The injection of cobra venom (2–20 mg.) into perfused organs (lung, liver) of dogs and monkeys causes the appearance in the venous perfusate of histamine, of protein and of a substance or substances which cause slow contraction and transient changes in the excitability of the guinea pig's gut. In the case of the liver, pigments are also set free. No histamine appears in the perfusate of envenomed monkey's liver, since this organ has a very low histamine content. The changes in the venous perfusate from the liver of dogs poisoned by intravenous injections of cobra venom are similar to those observed when the venom is injected into the isolated organ.

"2. Histamine, protein and liver pigments are liberated from the cells of perfused organs, but the substance (or substances) which causes slow contraction of the gut and subsequent changes in its reactivity is *formed* in the organs by the action of the venom.

"3. This substance is present in large amounts in extracts of envenomed organs; it is soluble in absolute methyl alcohol and heat stable. Pharmacological actions of alcoholic extract of envenomed monkey's liver ('envenomed liver') have been compared with those of cobra venom and of extract of lecithin treated with venom ('lysocithin').

"4. 'Envenomed liver' and 'lysocithin' cause slow delayed contraction of the guinea pig's jejunum and characteristic after-changes in reactivity to histamine and to acetylcholine. The effects of cobra venom are similar, but in this case the muscle is readily desensitized.

"5. 'Envenomed liver,' 'lysocithin,' and cobra venom contract the rat's jejunum, the normal guinea pig's uterus and the uterus poisoned by histamine. In the case of cobra venom the preparations are readily desensitized.

"6. On the isolated cat's heart 'lysocithin' causes changes in coronary circulation and strong reduction in the force of the beat; rapid

failure occurs and the heart ceases to beat in diastole or in midposition. Extracts of normal monkey's liver, which by themselves have no action, protect from the action of 'lysocithin,' and if the protection is not complete a gradual failure occurs similar to that caused by 'envenomed liver'; cobra venom (2-4 mg.) causes rapid failure and systolic contraction of the heart.

"7. Injected into the anterior chamber of the rabbit's eye, 'envenomed liver,' 'lysocithin,' and cobra venom cause opacity of the cornea and irregular alterations of its curvature. Extracts of normal monkey's liver are without effect.

"8. Injected intravenously into guinea pigs, 'envenomed liver' and 'lysocithin' cause symptoms resembling acute anaphylactic shock with the addition of haemorrhagic oedema of the lungs.

"9. Washed sheep's red corpuscles are immediately haemolyzed by 'envenomed liver' and 'lysocithin' but not by cobra venom\* nor by extract of normal monkey's liver; the latter has a protective action against haemolysis by 'lysocithin.'

"10. 'Envenomed liver' and 'lysocithin' injected into the perfused dog's liver cause output of protein, histamine and pigments; with repeated injections the output of histamine increases. These effects are closely similar to those produced by repeated injections of small doses of cobra venom."

In addition to the protection, or neutralization, provided by anti-venom serum against the toxic effects of clotting and proteolytic enzymes, and lecithinases of snake venom, antivenom neutralizes also the nucleic acid hydrolyzing enzyme or enzymes present in the venom.

## 2. Ricin

a. **Chemical Nature of Ricin.** Similar to snake poisons and bacterial toxins there are a number of plant poisons which are either strongly hemolytic or exercise hemagglutinating activity. They are ricin, abrin, crotin, curcin, robin, etc. Of these crotin and curcin are reported to be particularly active hemolytically, while ricin, abrin and robin are characterized by their agglutinating properties, hemolysis being produced only by the use of relatively larger amounts. The serological properties of ricin have been widely investigated.

\*Washed cells are devoid of free lecithin which is the necessary substrate for the enzyme of venom to produce the hemolytic "lysocithin."

Osborne, Mendel and Harris (1905) prepared a highly pure ricin of which 0.001 mg. per kg. was fatal to a rabbit. It was obtained by extracting the pressed cake of castor oil plant seeds with 10 per cent sodium chloride. The extract on dialysis yielded a globulin precipitate which was discarded. When the supernatant was treated with ammonium sulfate, ricin precipitated out. The precipitate was dissolved and reprecipitated with ammonium sulfate, followed by dialysis which gave them an albuminous substance with the above highly toxic properties. They established the protein nature of the toxin. They further found that 0.01 to 0.007 per cent ricin solution was effective in causing the agglutination of the red blood corpuscles of various animals. Karrer, *et al.* (1924) in an extensive study attempted to improve the purity of the ricin prepared by Osborne, *et al.*; but, they failed to obtain a purer preparation. Karrer *et al.* for purification purposes employed methods such as adsorption on kaolin, aluminum hydroxide and precipitation by metallic salts. Confirming the observation of Osborne, *et al.*, they also found that the loss of toxicity ran parallel with the proteolytic hydrolysis of ricin. In the proteolytic digest, part of the ricin was found to remain undigested which was isolated and shown to be biologically not different from the original ricin. Chemically the undigested ricin contained 2.5 to 3 per cent lower total nitrogen which was entirely accounted for by the possible splitting of the arginine guanidine group and thereby the conversion of arginine into ornithine groups. However, this change produced no decrease or loss of toxicity in the ricin molecule.

**b. Inhibition of Lecithinase and Hemolytic Activities of Ricin by Anti-Ricin Immune Serum.** In 1897 Ehrlich showed that the action of a solution of ricin *in vitro* on citrated blood causes clumping and the precipitation of the corpuscles. He further showed that this action could be completely done away with by previously mixing with the ricin definite quantities of anti-ricin serum. Mixtures of the toxin and antitoxin which did not clump were found by Ehrlich to be innocuous on injection into mice. His test tube experiments were further found to represent with much accuracy occurrences within the animal body.

Rehns (1902) reported that ricin adsorbed on red blood corpuscles was neutralized and removed with anti-ricin serum. The recovered ricin possessed all its agglutinating, antigenic and toxic properties. Conversely Madsen and Walbum (1904) reported that, depending

on the concentration of antiricin present in the ricin-antiricin neutral mixture, red blood corpuscles exercised the power of dissociating ricin from the neutral mixture and fixing it. Despite the great affinity between ricin and red blood corpuscles on one hand, and lecithinase activity of ricin on the other, the hemolysis does not appear to take place so readily. Pascucci (1905) on the other hand reported that ricin acting on a saline emulsion of lecithin produced a flocculent precipitate. The filtrate from the precipitate added to red blood cells produced immediate hemolysis.

Bertarelli (1926) also reported that ricin possesses lipase activity. The lipase activity was determined by titrating with standard alkali solution the acidity developed after ricin had acted on olive oil for two hours at 37°C. Immune rabbit or dog sera prepared against the ricin preparation exercised appreciable inhibition (25 to 30 per cent) on the lipase activity of ricin. He did not observe any inhibitory effect by immune sera on lipase activity of normal serum, or pancreas and liver extracts.

More definite information was obtained by Neuberg and Rosenberg (1907) and Neuberg and Reicher (1907) regarding the relation of the lecithinase activity of ricin to hemolysis. They compared the lipase and lecithinase activity of ricin with those of snake venoms and also of bee poisons, and found all of them to possess strong lipolytic activity. Investigating the lipolytic and hemolytic properties of the culture filtrates of various bacteria, they found that while the hemolytic filtrates of cholera vibrios, staphylococcus and meningococcus exercised weak lipase activity on olive oil, lecithin and castor oil were strongly hydrolyzed. Ricin likewise strongly hydrolyzed lecithin and castor oil. The hydrolytic mixture of lecithin was hemolytic. Neuberg, *et al.* (1907) reported that antiricin immune serum completely neutralized the lecithinase and lipase activity of ricin.

### 3. Bacterial Hemolysins

The mechanism of hemolysis by bacterial filtrates has until recently remained relatively obscure. The reason probably was the absence of sufficient chemical data as to the nature of hemolysins and their various chemical and physical properties. It is known that numerous pathogenic and non-pathogenic micro-organisms elaborate hemolytic



substances, but only pneumococcal and streptococcal hemolysins have been the subject of considerable investigation.

a. **Hemolytic, Dermonecrotic and Lethal Activities of Staphylococcal Toxin.** Kraus and Pribram (1906) reported that certain strains of *S. aureus* produced powerful toxins fatal to rabbits. The culture filtrates of this organism have been shown to exercise necrotic, lethal and hemolytic activities. Burnet (1929) stated that these three activities of staphylococcal exotoxin reside in the same substance. He believed in the presence of only one antibody, capable of neutralizing all three activities of the toxin: "It is particularly impressive that antitoxic sera prepared in almost all the possible ways showed a constant relationship between their antihemolytic and antitoxic powers." Levine (1939) also stated that the properties of staphylococcal toxin are those of a single substance. He found that 1 unit of U. S. Standard staphylococcus antitoxin exactly neutralizes the hemolytic, dermonecrotic and lethal properties of 0.00192 g. of dry staphylotoxin, quantitatively measuring the neutralization of toxic effects by standard antitoxic serum. Potency estimations of 10 commercial concentrated staphylococcal antitoxins were found to have the ratio of hemolytic: dermonecrotic: lethal potency values of 1.0:1.0:1.0.

*The pharmacological action* of staphylococcal toxin has been investigated by Kellaway, Burnet and Williams (1930). These studies showed that the toxin caused quick death of cats, hemorrhages in the lungs, pulmonary edema and distention of the right heart with blood. Intravascular hemolysis was a striking feature in these animals. Feldberg and Keogh (1937) reported that staphylococcal toxin produced the same effect on animal organs as lysolecithin and snake venoms. Staphylococcal toxin caused an output of histamine from the perfused lung of guinea pigs and cats. They stated that the results were comparable to those obtained with snake venoms. They believed that a mechanism similar to the liberation of histamine by staphylococcal toxin is involved in the action of other bacterial toxins.

The above findings in comparison with the action of snake venom suggest that the liberation of histamine in the animal organs by staphylococcal toxin might be related to the formation of lysolecithin by its action on tissue lecithin. Since, however, there is as yet no direct experimental finding that shows that the toxin acting on lecithin produces lysolecithin, the validity of the above assumption, based on



the analogy of effects between snake venom and lysolecithin on one hand, and staphylococcal toxin on the other, must await further direct experimental evidence.

**b. The Relation of the Lipase Activity of Staphylococci to Hemolysis.** In this connection it may be of interest to record here a few findings regarding the lipase activity of staphylococcal culture filtrates. This is true since there appears to be a possibility that the lipase and hemolytic activities may be related. Eijkman (1901) reported that a hemolytic *Staphylococcus aureus* exercised lipase activity, and expressed the view that the hemolytic activity of micro-organisms is due to an enzyme action. Orcutt and How (1922) isolated a staphylococcus from milk which was found to possess a thermolabile and extracellular hemolytic factor. This factor by itself was non-hemolytic, but acquired hemolytic power when it was allowed to act on cream, butter, olive oil and triolein. It did not render tributyrin, triacetin, nut butter, pork fat and fat-free milk hemolytic.

They also isolated a staphylococcus C from a lung abscess of a cow which manifested the same hemolytic power as the above. When a living culture or an etherized culture of the staphylococcus was permitted to stand with cream or other fat for several hours and then was heated to 100°C., the resulting fluid was capable of producing hemolysis. The "hemolysin" in the culture filtrate was not dialyzable. It was inactivated at 55°C.

Hemotoxin production in staphylococci was stated by McBroom (1937) to be definitely correlated with the extent and speed of reduction of methylene blue to the leucobase in the presence of glucose.

*Antibody Against Lipase of Acid-Fast Bacteria:* Sartory and Meyer (1947) have described the production of antilipase to the lipase of the Koch bacillus and *Mycobacterium phlei*. Lipases isolated from the bacteria were used to immunize male rabbits. The period of treatment at 5-day intervals was extended to 2.5 months. At the 80th day of immunization antilipase activity of the rabbit serum was tested using a saturated aqueous solution of tributyrin as substrate. At this date the antilipase titer was very high, and two months after the interruption of the injection of lipase, the serum of the rabbit showed still a detectable antilipase activity. The antilipase produced against lipase of acid-fast bacteria had no inhibiting action on normal pancreatic lipase or on the normal serum lipase of man or the young rabbit. The

antilipase activity of the serum of sensitized rabbits was stable to heating for 30 min. at 57° but not at 80°.

**c. Hemolytic Toxin of *Clostridium Septicum*.** Bernheimer (1944a) studying the relation of the lethal toxin to the hemolysin produced by *Clostridium septicum*, strain 44, reported that the hemolytic and lethal actions of crude toxin are functions of a single substance or that they are functions of two substances which have similar physical, chemical, and antigenic properties. This conclusion was based on the following observations: (1) the lethal activity of cultures is directly proportional to their hemolytic activity; (2) hydrogen peroxide diminishes to the same extent the hemolytic and lethal activities; (3) the hemolytic principal and the lethal toxin are adsorbed to approximately the same extent by charcoal, and kaolin; (4) erythrocytes remove the lethal activity as well as most of the hemolytic activity from the culture supernate; (5) both lethal toxin and hemolytic principle are partially destroyed in dilute solution at 36°C.; and (6) the anti-hemolytic capacity of antitoxic horse serum is directly proportional to the antilethal capacity. On the basis of the results obtained from kinetic study of the hemolytic reaction with respect to concentration, temperature and hydrogen ion concentration, Bernheimer (1944b) observed a resemblance to enzyme-catalyzed reactions, except that there was absent a clearly defined pH optimum.

#### 4. Pneumococcal Hemolysin

Pneumococcal hemolysin is found in the lytic extract of pneumococci that causes the death of guinea pigs on intravenous injection. It is destroyed by the action of trypsin. Its activity is prevented by the presence of minute amounts of cholesterol. Injected into rabbits and sheep, the hemolysin produces antihemolysin, which is species specific. Hewett and Famulener (1922) inferred, and Avery and Neill (1924) confirmed, that the hemolysin was an intracellular product liberated from the autolyzed organisms. The latter investigators made further observations to the effect that the hemolytic extracts undergo auto-oxidation. Neill (1926) later showed that auto-oxidized inactive hemolysin is reducible and thereby rendered reactive by sodium hydro-sulfite.

Schwachmann, Hellerman and Cohen (1934) stated that the activ-

ity of pneumococcal hemolysin appears to be controlled by the oxidation-reduction state of certain thiol groupings in its structure. An attempt has been recently made by Cohen, Halbert and Perkins (1941) to obtain pneumolysin in purified form. In this work, the authors carried out extensive experiments on the optimal conditions of hemolysin production, method of purification, and chemical and physical properties of the relatively purified product. It could be precipitated at pH 4.0 without losing its activity. The purified substance had a nitrogen content of 13 to 15 per cent. It gave protein tests and contained 1.0 to 2.0 per cent phosphorus. Commercial trypsin, chymotrypsin, pepsin and papain destroyed the hemolytic activity. The activity of hemolysin was unaffected by repeated extraction with dry benzol, pyridine, chloroform, acetone or petroleum ether at 20° to 25°. By the ultracentrifugal method, the lysin's rate of sedimentation seemed to be appreciably faster than that of egg albumin (mol. wt. of about 45,000) and somewhat slower than that of hemoglobin (mol. wt. about 66,000).

The hemolytic unit, H.U., was arbitrarily set at the 50 per cent hemolysis of 4 ml. of a 1 per cent suspension of red cells (or the complete hemolysis of 2 ml. of the cell suspension). The reagent red cells were rabbit erythrocytes washed thrice by centrifuging in the cold with M/15 phosphate of pH 7.6 and resuspended in the proportion of 1 volume to 99 of the same buffer.

The activity of the purified hemolysin was measured by Cohen *et al.* and it was found that the most active preparations contained 6000 H.U. per mg., or 1 H.U. per  $2.4 \times 10^{-5}$  mg. N (or  $1.58 \times 10^{-4}$  mg. protein); the average sample contained 2000 H.U. per mg.

Gelatin, casein and egg albumin exercised no inhibitory effect on the lysin. The apparently clear "hemoglobin" solution, prepared by laking and centrifuging washed rabbit erythrocytes, inactivated hemolysin; but Seitz-filtered hemoglobin solution was ineffective. Benzol extraction also removed the inhibitory substance from the unfiltered "hemoglobin" solution. A suspension of 2 micrograms of washed stromata obtained from laked rabbit blood inactivated or adsorbed 16 H.U. of hemolysin (2.37 microgram) in 15 minutes. The same stromata exhaustively extracted with benzol after drying did not at all affect, even in amounts up to 250 micrograms, the activity of 18 or 5 H.U. of lysin.

Tests with pneumococcal hemolysin for proteolytic and phosphatase activity were negative, and tests for lipolytic activity were stated to be inconclusive. It did not cause detectable turbidity or a flocculation when mixed with egg-yolk solution. Hemolytic activity was unaffected after treatment with 0.05 M oxalate, citrate or fluoride. They stated that all of their assays showed practically stoichiometric relations between the amount of lysin and the number of cells, within the titration range.

On the basis of the above observed facts, the statement was made that the action of the lysin upon the red cell, or upon the usual substrates, thus far indicated that it possessed no enzymatic activity.

It is possible to interpret the findings of these investigators in another way, however. The observed stoichiometric relationship might be due to a combination between a "cholesterol-like" inhibitor substance, produced as one of the hemolytic reaction products, and the hemolysin.\* According to this hypothesis, the inhibitor is present in the intact cell, but as part of the cell is ineffective to inhibit the lysin. Its inhibitory action arises as a consequence of the rupture of the cell by the hemolysin. The combination of such reaction products and the catalyst is often irreversible and obeys the mass action law. For example,  $\beta$ -maltose as part of the starch molecule has no inhibitory action on  $\beta$ -amylase. However, following the hydrolysis of starch by amylase,  $\beta$ -maltose is liberated and only then exercises an inhibitory action on amylase. The inhibition of pneumococcal hemolysin, fol-

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\*In this connection the results of a comprehensive study by Ponder (1946) on the mechanism of the inhibition of non-enzymatic hemolysis by saponin, sodium taurocholate, or sodium glycocholate might be of interest. He reported the following observations:

(1) The suspension medium of a thrice washed red cell suspension contains inhibitory substances which render inert a small quantity of lysin, so far as the hemolytic effect is concerned.

(2) On the addition of the lysin to the cell suspension, a further quantity of lysin is rendered non-hemolytic within the short time necessary for the separation of the cells from the bulk phase of the system, and before any lysis takes place. This quantity is several times greater than that found in (1).

(3) The colorimetric measurements show that the quantity of chromogenic material in the bulk phase, after contact with the cells, is substantially the same as that present initially, and that no appreciable quantity of the lysin initially present accumulates in increased concentration at the red cell surfaces.

According to Ponder (1943) the inhibition of saponin lysis by plasma seems to be somewhat as follows: About 35 per cent of the inhibition is due to cholesterol, about 15-25 per cent to the globulins, and the rest to "enhancing effects," particularly by lecithin on the cholesterol inhibition. The latter are very complex.



lowing the hemolysis of erythrocytes and the liberation of certain reaction products, may be comparable to the inhibition of amylase by  $\beta$ -maltose as a hydrolytic product of starch.

The substrate for hemolysin according to this interpretation is the red cell proper. The function of hemolysin as catalyst is to disorganize the highly organized cell, and it does this without being inhibited during the process of hemolysis by the "inhibitors" present in the intact cell. The inhibitors, believed to be cholesterol (and lipids), are liberated as a result of catalytic disorganization of the cell.

The combination between the hemolysin and cholesterol (one H.U.=3 to  $9 \times 10^{-5}$  mg. N and  $1.5 \times 10^{-5}$  mg. of cholesterol, Cohen, *et al.* 1940) does not appear to produce chemical changes in their constitution. For, the combination cholesterol-lysin, extracted during the most rapid and effective stage of union by benzol, yields a product which is actively hemolytic again (recovery from 1 to 16 per cent). These investigators stated that "Despite the low recoveries, it seems significant that the inactivating cholesterol is removable by simple extraction." These findings show that the stoichiometrical inactivation of lysin by cholesterol is not due to an irreversible chemical degradation of the reactants. This type of inactivation by cholesterol is the common characteristic of inhibitors produced during a reaction catalyzed by an enzyme.\*

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\*Bernheimer and Cantoni (1945) reported that the preparation containing oxygen-labile hemolysin of *Streptococcus pyogenes* induces systolic contracture, the contracture usually developing only after the second of two administrations of the preparations. A single application of the streptococcal preparation sensitized the heart to a second application. The cardiotoxic factor was found to be identical with the oxygen-labile hemolysin of streptococci. The capacity of normal and of immune sera to neutralize the cardiotoxic action paralleled the antihemolytic potency of the sera. Cantoni and Bernheimer (1945) found that the sensitization depends upon the release from the isolated heart of a substance which inhibits the action of the cardiotoxin. The inhibitor is released upon the first (sensitizing) administration of cardiotoxin, but not upon the second (contracturing) administration. The released inhibitor neutralizes the lethal factor present in the streptococcal preparation employed. The inhibitor is thermostable, 10 minutes at 70°C. chloroform-soluble and non-dialyzable, which may suggest that it is a lipid-like substance, or cholesterol. Hewitt and Todd (1939) made the observation that, in the absence of protein, streptolysin S is powerfully inhibited by lecithin. Humphry (1949) reports that anti-streptolysin S is not an antibody in the accepted sense, and its nature varies between species, and even between individuals. He described two types of antistreptolysin S. *One*: Lipoprotein loose complexes represented by (a) materials extractable (thus removing inhibitory activity) with ether, and (b) stable lipoprotein complexes, the activity of which are removed by ether after digestion of denatured material. *Two*: Non-specific proteins, the activity of which is destroyed by prolonged peptic digestion.



**a. Number of Pneumococcal Hemolysin Molecules Required to Hemolyze One Red Blood Cell.** The plasma of human blood amounts to 55 per cent by volume; the cells make up the remaining 45 per cent. There are  $5 \times 10^9$  red blood cells in one ml. of blood. One *hemolytic unit*, H.U., of pneumococcal hemolysin was defined by Cohen, Halbert and Perkins (1941) as that amount of hemolysin capable of 50 per cent hemolysis of 4 ml. of a 1 per cent suspension of washed red cells (or 2 ml. of suspension when complete hemolysis is considered).

One ml. of whole blood contains  $5 \times 10^9$  r.b.c. Since 45 per cent of the whole blood consists of red cells, 0.45 ml. of undiluted red cells therefore contains  $5 \times 10^9$  r.b.c., or 1 ml. of undiluted red cells contains  $1.11 \times 10^{10}$  r.b.c. Two ml. of 1 per cent suspension of red cells should therefore contain  $1.11 \times 10^{10} / 100 \times 2 = 2.22 \times 10^8$  r.b.c. Therefore 1 unit of hemolysin is capable of completely hemolyzing  $2.22 \times 10^8$  r.b.c.

The purest hemolysin contained 6000 H.U. per mg. Therefore  $2.22 \times 10^8 \times 6 \times 10^3 = 1.332 \times 10^{12}$  red blood cells are hemolyzed by 1 mg. of hemolysin.

The data indicate that the hemolysin might have a molecular weight of about  $5 \times 10^7$  in mg., or ( $5 \times 10^7$  mg. =)  $6.06 \times 10^{23}$  molecules.

One mg. of hemolysin contains  $1.21 \times 10^{16}$  molecules ( $6.06 \times 10^{23} / 5 \times 10^7$ ).

This shows that  $9.09 \times 10^3$  molecules ( $1.21 \times 10^{16} / 1.332 \times 10^{12}$ ) of lysin are required for the hemolysis of one red blood cell.

If we calculate the ratio of the weight of hemolysin to the weight of red cells it hemolyzes we will have the following relationship.

The sp. gr. of red blood cells is 1.1, or 1 ml. of red cells weighs 1.1 g.

One ml. of undiluted red cells, as shown above contains  $1.11 \times 10^{10}$  r.b.c.

One r.b.c. weighs therefore  $1 \times 10^{-10}$  g. ( $= 1.1 / 1.11 \times 10^{10}$ ).

One mg. of hemolysin hemolyzes  $1.332 \times 10^{12}$  r.b.c.

One mg. of hemolysin hemolyzes  $1.332 \times 10^2$  g. ( $= 1.332 \times 10^{12} \times 1 \times 10^{-10}$ ) or  $1.332 \times 10^5$  mg. r.b.c.

Since only 40 per cent of the r.b.c is total solids the ratio on a dry weight basis is  $133,200 \times 0.4 = 53,280$ , or 1:53,280.

However, if we consider the stroma of the red cells as a closer approach to the true substrate, and since the stroma represents about 10

per cent of the mass of the red blood cells, the weight-to-weight ratio will be 1:5,328 (dry basis). This, of course, does not take into consideration the fact that the hemolysin used is only partially purified. This relationship becomes easily understandable when the activity of hemolysin is considered as one of catalysis. This interpretation appears to be corroborated by the studies of Herbert and Todd (1941) which will be discussed below.

**b. A Study of the Antipneumolysin Titer of the Sera of Pneumonia Patients.** In a comprehensive study on pneumolysin, Oker-Blom (1948) reported the following findings. Sera of rabbits immunized with streptolysin showed pronounced increase of antistreptolysin values, but increased pneumolysin values could not be demonstrated in the same sera when titrated with pneumolysin. Normal human sera showed approximately the same antipneumolysin titer with pneumolysin from type 2 as from type 3 pneumococci. Sera of pneumonia patients showed twice as high titers as normal sera. The maximum increase of the antipneumolysin titer reaches a peak on the 18th to 23rd day from the onset of the disease, followed by a decline. A systematic study showed that there is a relation between an increased cold-agglutinin titer and increased antipneumolysin titer. There was, however, no constant relation between the periods of increased antipneumolysin and increased cold-agglutination titer (for earlier findings see White, 1938).

## 5. Streptococcal Hemolysin

Smythe and Harris (1940) found that *Streptococcus hemolyticus* Strains: 1685 M, Type I (Griffith); 1048 M, Type VI (Griffith); C203, Type I (Griffith). N.Y. 5, Wadsworth; B<sub>3</sub>S; and 1685 G (not fatal to mice) produced the same hemolysin. The hemolysin was inactivated by proteolytic digestion with trypsin, pepsin and papain. Boiling destroyed it immediately, and a temperature of 56° irreversibly inactivated it within a few minutes. It was completely precipitable with saturated ammonium sulfate and partially with three volumes of alcohol, acetone or dioxane from the broth culture filtrates. The active substance was readily soluble in glycerol. The cupric salt precipitates of hemolysin were reactivated with sodium pyrophosphate. Alum also precipitated the active fraction in a recoverable manner. It was

stable at pH 4.0 to 8.5. The minimal solubility, as in pneumococcal hemolysin (Cohen, *et al.*, 1942) in a concentrated broth, was at pH 4.0 to 4.5.

*The unit of hemolytic activity* was taken as the smallest amount of the solution tested that completely hemolyzed 0.5 ml. of a 2.5 per cent suspension of sheep red blood cells in 30 minutes at 37° in a final volume of 1.0 ml. The purified hemolysin contained 0.001 mg. of nitrogen per hemolytic unit. The hemolysin produced in a synthetic medium on purification contained 0.0005 mg. of nitrogen per hemolytic unit. The dialyzed hemolysin contained 15.3 per cent nitrogen, 1.9 per cent sulfur and approximately 0.1 per cent phosphorus. Tests for inorganic sulfate were negative. Tests for organic sulfate showed 1.0 per cent. The inactive preparations of hemolysin gave negative nitroprusside tests, but when they were treated with potassium cyanide, the test became positive. Using cysteine as a standard a colorimetric determination showed that only a part of the total sulfur was present as -SH.

The hemolysin was reversibly inactivated by oxidizing agents such as oxygen, hydrogen peroxide, iodine and potassium ferricyanide. Such oxidized hemolysin could be reactivated by sodium hydrosulfite or by cysteine, glutathione, thioglycollic acid, sodium bisulfite, hydrogen sulfite or potassium cyanide. Sodium thiosulfate also had a slight activating effect. The effect of potassium cyanide, in comparison to cysteine, was definitely less. Only a very slight activation of hemolysin with ascorbic acid or with ascorbic acid plus iodide was observed. Hydrogen and palladium-asbestos had no activating effect. The leuco forms of several dyestuffs were also unable to activate the hemolysin. Rosindulin G.G., which has a very negative potential, in no case caused the activation of inactive hemolysin. Equally negative results were obtained with methylene blue, indigo disulfonate, indigo tetrasulfonate and pyocyanine. Activation with methyl viologen, although far from complete, was very definite.

The hemolysin was also inactivated by cuprous oxide, phenylmercuric chloride, phenylmercuric nitrate, alloxan, maleic acid, iodoacetic acid, iodoacetamide and cholesterol. The inactivation caused by each of these, except the last, was at least in large part reversed by thiol compounds.

The results with the activating and inactivating agents suggested

that the hemolysin contains a  $-SH \rightleftharpoons S-S-$  oxidation-reduction system, and that this system must be in the  $-SH$  form in active hemolysin. However, the high content of sulfur, only a part of which existed as  $-SH$  groups, suggested another possible rôle for this element.

The partially purified hemolysin was neutralized by various antisera, as was the untreated supernatant broth of cultures of hemolytic streptococci. This neutralization was independent of purification or reduction of the hemolysin, and was not accompanied by precipitation, in the available range of concentration of the reagents. For a discussion of the observations regarding the *in vitro* formation of streptolysin S the reader is referred to an article by Bernhermer (1949).

a. **Streptolysin O.** Herbert and Todd (1941) reported the following findings from a comprehensive study of the reversibly oxidizable streptolysin of Group A hemolytic streptococci. This hemolysin is produced by most strains of Group A streptococci when grown in a serum-free medium such as glucose-bicarbonate-phosphate-broth; it is also produced by Group C strains from human infections and by Group G strains, but not by streptococci of other groups. The streptolysins produced by all types and strains of Group A streptococci are serologically identical.

Streptolysin O was active only in the presence of certain reducing agents, such as thiolacetic acid, and it was found necessary to add these to the reaction system. The purification of the hemolysin involved fractionation with ammonium sulfate, adsorption and elution from calcium phosphate gel, followed by adsorption and elution from alumina C $\gamma$ . The purified hemolysin contained 3050 hemolytic units, H.U., per mg. by dry weight; or 1 H.U. contained 0.000,05 mg. of nitrogen, a 10-fold greater purification than that of Smythe and Harris (1940). The purified hemolysin contained: C, 46.7; H, 6.8; N, 16.8; S, 2.34; P, 0.053; ash, 3.6 per cent. The total carbohydrate was 2.6 per cent. It gave the usual protein tests. Boiling for two minutes caused complete and irreversible inactivation; the same result was obtained on treatment with strong acids and alkalis.

The purified preparations of streptolysin O were neutralized by antistreptolysin O to the same extent as the crude preparations. They were neutralized by antisera to *Cl. welchii*  $\theta$ -hemolysin, but not by antisera to *welchii*  $\alpha$ -hemolysin. The  $\alpha$ -hemolysin of *Cl. welchii* hydrolyzes lecithin, but streptolysin does not give this reaction. No trace



of opalescence was observed after incubating a strong solution of streptolysin for 24 hours at 38° with the lecitho-vitellin solution.

The purified streptolysin O preparations were toxic to mice on intravenous injection. The minimum lethal dose was about 135 H.U. or 0.044 mg. of the preparation. The streptolysin O had a much greater lethal effect when injected in an activated form, e.g. together with cysteine, although cysteine alone had no harmful effect. The authors suggested that *in vivo* its action is similar to that *in vitro*.

Streptolysin O was stated to be different from the erythrogenic toxin, streptolysin S, leucocidin, fibrinolysin and the diffusing factor (hyaluronidase).

It was inactivated by oxidation, and the inactivation was reversed by all the compounds containing the -SH group. Compounds containing the -S-S- group, ascorbic acid and ferrocyanide had no activating effect at all. A medium degree of activation was achieved by potassium cyanide and sodium thiosulfate. These facts suggested that the hemolysin molecule containing the -SH group is active; its oxidation to the -S-S- group inactivates it. This confirmed the observations by Smythe and Harris (1940).

The hemolytic activity was greatest at pH 6.5 and decreased markedly on both sides of this optimum pH. The optimum temperature of the hemolytic activity was 38°. At 0° the activity was 3 per cent of the optimal activity.

At 0° hemolysin adsorbs on red cells without hemolysis for half an hour. The supernatant of the centrifuged cell-hemolysin mixture is free from hemolysin. On suspending the centrifuged cells in fresh saline and incubating at 38°, they are rapidly hemolyzed. The results were interpreted to show that the adsorption of streptolysin on the red cell surface is a different reaction from the actual process of lysis, in which a chemical process is probably involved, since it is inhibited, while adsorption processes are increased, by low temperatures.

According to Herbert (1941) when small amounts of hemolysin were added to the system only a small percentage of the red cells present were hemolyzed; the smaller the number of red cells present, the greater the amount of hemolysis. This means that for each individual red cell, a certain critical amount of streptolysin must be adsorbed on its surface to cause hemolysis in a given time (see the discussion on pneumococcal hemolysin); if less than this amount is present, no



hemolysis takes place. The hemolysis of a single red cell, it was thus presumed, is an "all-or-none" process.

One hemolytic unit of streptolysin O was found to contain  $5 \times 10^{-5}$  mg. of nitrogen, which compared satisfactorily with the activity of pneumococcal hemolysin containing  $2.4 \times 10^{-5}$  mg. N per 1 H.U. Our calculation showed that  $9.08 \times 10^3$  molecules of pneumococcal hemolysin were needed to hemolyze one red blood cell. Assuming that the molecular weights of streptolysin and pneumolysin are comparable the same number of molecules of the hemolysins should produce an equal hemolytic effect. On a weight-to-weight basis the activity of streptolysin will thus show a ratio of about 1:53,280 (see page 323).

Herbert and Todd stated that 0.02 M iodoacetic acid had no effect on streptolysin O when kept with it for 20 minutes at room temperature, and only a slight effect (22 per cent inhibition) at 38° C. With 0.02 M iodoacetamide, which is considered to react with protein -SH groups more readily than iodoacetic acid, only partial inhibition (66 per cent) resulted. These results only partially agree with those of Smythe and Harris (1940).

**b. Probable Enzymic Nature of Bacterial Hemolysins.** The reactivation of inactive hemolysin by hydrosulfite, cysteine or a number of reducing agents makes it resemble a number of enzymes which are similarly affected by oxidation and reduction. These enzymes, urease, arginase, papain, cathepsin, succinic dehydrogenase, triosephosphate dehydrogenases are strongly inhibited by low concentrations of iodoacetic acid. Maschmann (1937) also found that the clupeinase activity of a toxin preparation of *Cl. welchii* was activated by cysteine, and while the proteolytic filtrates of *vibrion septique* and *B. botulinus*, types A and B, had no action on clupein, in the presence of cysteine they were rendered strongly proteolytic. The findings of Maschmann on these enzymes are, therefore, in agreement with the findings on the properties of hemolysins originating from bacterial filtrates.

Inactivated streptolysin O is not adsorbed by red cells, while the active form is, and it is suggestive that the -SH groups are necessary to attach the hemolysin to the red cell. This, however, cannot be too strongly supported in view of the fact that potassium cyanide, the activator of many of the above enzymes, fails to activate streptolysin O appreciably. Furthermore, streptolysin unlike these enzymes is resistant to the action of iodoacetic acid.

On the basis of their study, Herbert and Todd stated that the simplest explanation is that streptolysin O is an enzyme which attacks some constituent of the red cell membrane. Its protein nature, its activation by -SH compounds and many of its properties all suggest this. So far, however, no enzymic function has been found for it. This particular statement by the authors ignores the fact that the red cell is the substrate for their hemolysin. (It is not necessary that the substrate be a small crystalline or a large colloidal molecule, such as proteins or starch.) They further stated that the exceedingly small doses in which all bacterial toxins work suggest that their action must be catalytic.

For results of experiments in animals on the lethal effects of the oxygenlabile hemolysin see Bernheimer and Cantoni (1947), and for a comparison with saponin, Cantoni and Bernheimer (1947).

## I. ANTIBODY AGAINST NUCLEASES, UREASE AND PENICILLINASE

### 1. Antibody Against Ribonucleases

**a. Liberation of Adenyl Compounds from Perfused Organs Treated with Cobra Venom.** The power of snake venom to hydrolyze nucleic acid appears to be responsible for the following facts. Kellaway and Trethewie (1940a, 1940b) found that tissue injury by cobra venom in isolated perfused tissues causes the liberation not only of protein, pigments, histamine and the formation of a slow-reacting substance and lysolecithin, but also the liberation of adenyl compounds together with enzymes capable of inactivating them. The primary effect of adenylic acid on the heart of the rabbit causing a sinus bradycardia was stated to be the central feature of the action of cobra venom on the heart of the intact rabbit when venom was injected intravenously. The action of the venom on the heart of the dog was likewise found to accord with those produced by the injection of adenylic acid.

The greater part of the cardio-depressant activity in the perfusate from the perfused liver was found in that collected during the first three minutes after the injection of cobra venom. The proportion of adenyl compounds estimated in the perfusate increased when this

was treated at 95°C., or when Tyrode solution containing 0.025 N NaCN was used as the perfused fluid. A temperature of 95°C. or NaCN were found to inhibit the enzymes which inactivated the adenylyl compounds. While the perfusates from normal tissues were found not to contain either adenylyl compounds or the inactivating enzymes, the perfusate of the organs of rabbit and cat treated with cobra venom were found to contain free adenylyl compounds and the enzyme inactivating it.

**b. Antibody Against Nucleic Acid-Hydrolyzing Enzymes of Snake Venom.** Delezenne and Morel (1919) reported that snake venoms hydrolyzed both yeast and thymus nucleic acids and that the enzymes responsible for this effect were completely neutralized by anti-venom serum.

A carefully neutralized saline solution of yeast nucleic acid was treated with 0.1 per cent saline solution of snake venom and incubated at 50°C. In the presence of cobra venom, for example, nucleic acid lost its precipitability with hydrochloric acid. Thymus nucleic acid, which is very gelatinizable and solidifies in the cold, lost this property following the action of snake venom. They found that the time required for the unhydrolyzed thymus nucleic acid to gelatinize in the cold was proportional to the amount of the venom added. After a few hours incubation with the venom, thymus nucleic acid lost this property completely, and it did not assume viscous consistency even at ice-water temperature.

On the other hand, reaction mixtures neutral to phenolphthalein became markedly acid to litmus as the result of the formation of phosphoric acid and sodium acid phosphate. The development of acidity, followed by titration with alkali, was found to progress with the enzyme hydrolysis. The titration curve was typical of an enzyme reaction. The enzyme activity of the venom was completely inhibited in the presence of an optimal amount of antivenom specific serum.

They observed that the venom of colubrids, which possesses most marked general toxicity, was also the one which most readily hydrolyzed nucleic acid. In contrast, the venom of viperides, which was stated to be much less active, was found also not to exercise hydrolytic activity on nucleic acid under the experimental conditions employed.

**c. Inhibition of Ribonuclease by Homologous Anti-Serum.** Kunitz (1940) isolated a crystalline protein from beef pancreas capable

of hydrolyzing d-ribonucleic (yeast) acid. It had a molecular weight of about 15,000. Smolens and Sevag (1942) found that this enzyme injected into rabbits intramuscularly and intravenously elicited the formation of specific antibodies. Antibody was produced against crude and five, six and eight times crystallized enzyme preparations. In precipitation tests, immune sera prepared by different routes of injection reacted against antigen dilutions up to one million. Ordinarily there persisted a prozone with some antisera as high as to 1:100,000 antigen dilution. However, several antisera were prepared which did not show any antigen prozone.

In control experiments antisera prepared against cattle serum reacted with cattle serum in dilutions up to 31,000 without any prozone. Cattle antisera did not react with any of the preparations of ribonuclease. The cattle serum, likewise, gave no reaction against anti-ribonuclease sera. Normal rabbit sera when tested against the various ribonuclease preparations gave no reaction in any case.

The amount of the purified enzyme preparation in the antigen-antibody precipitate was determined by analyzing the precipitates for nitrogen. It was found that the antigen-antibody ratio was about 1:28 (1:40,000 antigen dilution). The experiments carried out under these conditions showed that the homologous antibody inhibited the d-ribonuclease activity from 10 to 30 per cent.

## 2. Neutralization of the Toxic Action of Crystalline Urease by Its Homologous Antibody

Details of the production and certain properties of immune serum against urease were described in Part I of this treatise. The physiological significance of anti-urease immunity will be discussed here further. The fact that urease, like toxins, is extremely poisonous to animal organisms has rendered it, in the hands of Sumner and Kirk, a valuable tool to study various phases of anti-enzyme immune reactions. The toxicity is due to the action of urease on the body urea producing fatal ammonia poisoning.

Kirk and Sumner (1931, 1934), Sumner and Kirk (1932) and Sumner (1937) showed that as little as 0.15 mg. (20 units of crystalline urease) is fatal to a rabbit of 2 kg. body weight. Death occurs when the blood ammonia has reached a concentration of 5 mg./100 ml.



Urease is completely inactivated by formalin; formalinized urease is non-toxic, and produces no antiurease on injecting into rabbits. A few seconds contact with 0.05 N hydrochloric acid destroys it in such a way that immediately after neutralization it does not produce a reaction with antiurease.

The toxic effect of urease was completely abolished when a rabbit was first given 90 units of antiurease followed three hours later by 90 units of urease. A second rabbit similarly treated with antiurease was also protected against 80 units of urease. Two rabbits which did not receive antiurease succumbed within five hours.

Six two-kilogram rabbits were injected intraperitoneally with 65 to 70 units of urease. After one and a half to two hours, the rabbits were totally paralyzed. Each rabbit was then given by ear vein 80 units of antiurease. Four of the rabbits showed immediate improvement, and became normal within one hour; the other two paralyzed rabbits could not be saved. In another experiment, 30 units of antiurease injected into each of two guinea pigs protected them against 15 units of urease injected similarly 90 minutes later. The control animal not receiving antiurease died. Hen antiurease serum, like that of immune rabbits, inhibited urease toxicity and protected rabbits from fatal doses of urease.

The studies by Kirk and Sumner (1931) thus showed that the fatal effect of urease in rabbits and guinea pigs was neutralized by antiurease unit for unit, in a manner similar to toxin-antitoxin neutralization reactions *in vitro* and in animals.

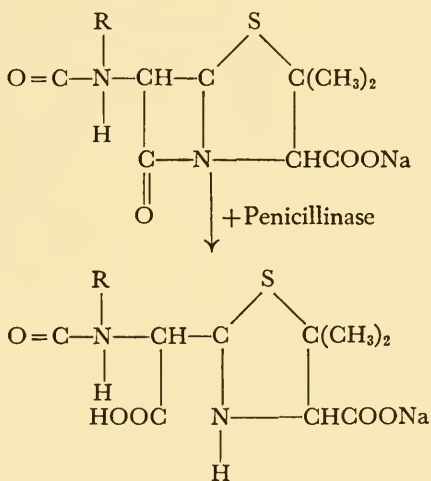
### 3. Antibody Against Penicillinase

Penicillinase is an enzyme that destroys penicillin (Abraham and Chain, 1940). It is produced by a variety of bacteria (Bondi and Dietz, 1944; 1946; Gilson and Parker, 1948).

Partial purification of penicillinase obtained from *Bacillus cereus* (Housewright and Henry, 1947a), and a comprehensive study involving the development of a manometric method of assaying penicillinase and penicillin and the kinetics of penicillin-penicillinase reaction (Henry and Housewright, 1947) is reported. Penicillinase is not a copper or iron enzyme. It is a protein or has a protein component essential for activity. Neither free amino groups nor sulfhydryl groups were



stated to be essential for enzymatic activity. It was found to be fairly resistant to oxidation but susceptible to reduction. The pH optimum of penicillinase at 36° was found to be approximately 7.2. It manifested a high degree of specificity for the configuration of the basic penicillin molecules. Penicillinase had no action on xanthine, adenine sulfate, guanine, riboflavin and uracil. These substances contain the configuration which is present also in penicillin. The action of penicillinase on penicillin results in the formation of a carboxyl group from the carbonyl group which is adjacent to the ring nitrogen. The carboxyl group which is formed reacts with bicarbonate liberating carbon dioxide which is measured manometrically.



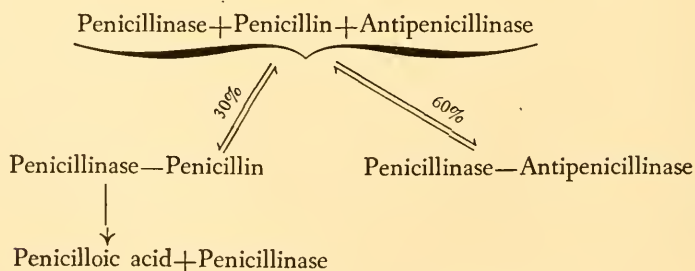
Perlstein and Liebman (1945) reported that 4000 units of penicillin were protected by antipenicillinase immune serum from inactivation by as high as 100 units of penicillinase. In the control series with normal serum or saline only 25 units of penicillinase were sufficient to inactivate 4000 units of penicillin in one hour at 37°C. They postulated the formation of a penicillin-plasma protein complex which protects penicillin *in vitro* from destruction by penicillinase.

Housewright and Henry (1947) remarked that such a postulate is not necessary since simple combination of penicillinase and antipenicillinase (antibody) should prevent destruction of penicillin, which is in accord with our view.

Housewright and Henry (1947b) immunized rabbits with dialyzed

penicillinase by injections on alternate days for five weeks. Precipitin reactions occurred in immune serum dilutions through 1:56. Inhibition tests were performed either by holding the penicillinase concentration constant and diluting the sera, or by keeping the concentration of sera constant and diluting the penicillinase. The enzymatic action of penicillinase was lost completely after contact for one hour at 37° C. with immune serum dilutions as high as 1:112. A partial loss was observed with serum dilutions of 1:224, 1:448 and 1:896. There was no observable inhibition with normal rabbit serum. There was no loss in enzyme activity when the enzyme, horse serum, rabbit antihorse serum, and saline were mixed and allowed to stand for one hour at 37° C. In manometric measurements, it was found that penicillinase incubated at 37° C. for one hour with antipenicillinase lost about 90 per cent of its activity. Antipenicillinase was found to combine with the enzyme almost immediately when it was introduced after allowing the penicillin-penicillinase reaction to proceed for 15 minutes. Controls indicated that there was no CO<sub>2</sub> retention by the concentration of serum used.

The data presented by Housewright and Henry (1947b) show that the substrate and the inhibiting antibody compete for the active site of penicillinase. When penicillinase and antipenicillinase react first, penicillin is incapable of counteracting or displacing the antibody from its combination with the enzyme; under this condition the inhibition of the enzyme is about 93 per cent. On the other hand, when the antibody was added to the enzyme-substrate reaction system, it was able to displace the substrate partially from the active site of the enzyme. Under these conditions, the inhibition at the end of 110 minutes was about 59 per cent. At this period, the curve indicated flattening and therefore two states of equilibrium.



Attention may be called to a basic principle in enzyme reactions that all reversible inhibitors whose action is upon the same enzyme center as normally would combine with substrate molecules are *necessarily* competitive inhibitors. The *degree* of competition will naturally vary, but whether it be considerable or very slight, there is no basic difference in the kinetic mode of action.

## J. IMMUNITY AGAINST THE ENZYMATIC ACTIVITIES OF BACTERIAL TOXINS

### 1. Bacterial Toxins

Effects on animal organs *in vitro* by snake venom poisons were found to be accounted for by the formation of hemolytic lysolecithin which was believed to afford a satisfactory basis for their effect *in vivo*. Neill and Fleming (1927) reported that sterile filtrates of *Cl. botulinum* exercised a lipase activity. Fleming and Neill (1927) found that *Cl. welchii* likewise exercised the power of hydrolyzing tributyrin. They did not, however, mention whether or not these filtrates were also active on lecithin. It is due to the careful work of MacFarlane and Knight (1941) that we have a clear picture of the relation of the lecithinase activity of the toxins of *Cl. welchii* to their hemolytic and other toxic properties. Since this work toxins have been obtained in crystalline form and their properties determined. These and other related studies will be discussed below.

a. **Type A Toxin of *Clostridium botulinum*.** Lamanna, *et al.* (1946a, 1946b) described a method for the isolation of a highly toxic needle-shaped crystalline protein from a type A culture of *Clostridium botulinum*. Electrophoretic analysis showed homogeneity and a mobility of  $2.75 \times 10^{-5} \text{ cm}^2 \cdot \text{volt}^{-1} \cdot \text{sec}^{-1}$ . The sedimentation diagram showed a single symmetrical boundary in the ultracentrifuge, yielding a value of  $S_{20} = 17.3$  Svedberg units. The diffusion constant by the refractometric scale method was  $2.14 \times 10^{-7} \text{ cm}^2 \cdot \text{volt}^{-1} \cdot \text{sec}^{-1}$ . From these data a molecular weight of 900,000 was calculated (Putnam, Lamanna & Sharp, 1946). It contained 14.3 per cent nitrogen. The Molisch test was negative. The pure toxin is a protein with the solubility properties of globulin. One MLD (mouse) contained  $4.2 \times 10^{-9} \mu\text{g}$  nitrogen, or  $3 \times 10^{-5}$  mg. of toxin. The data suggest that there are  $2.1 \times 10^7$  mole-

cules/LD50. This figure makes botulinus toxin the most potent poison known. Amino acid analysis of the crystalline toxin (Buehler, *et al.*, 1946) showed 14 amino acids of which 10 amino acids are necessary in animal nutrition. It contained 14.9 per cent glutamic acid, the highest value obtained for any amino acid. Qualitative tests for glycine, alanine, proline and hydroxyproline were negative.

Using a different technique of isolation Abrams, *et al.* (1946) obtained the same toxin in crystalline form in 0.10 to 0.30 saturated ammonium sulfate at 4°. An isoelectric point of pH 5.6 and a total nitrogen of 14.1 per cent was reported.

**b. Biological Action of Botulinus Toxin.** In contrast to other bacterial toxins, botulinus toxin is effective when administered by mouth. This is probably due to the fact that it is relatively resistant to the action of pepsin and trypsin. Bishop and Bronfenbrenner (1936) have reported that this toxin acts specifically on the myoneural junctions. Torda and Wolff (1946) using impure toxin reported that small amounts of the toxin decrease the synthesis of acetylcholine in both *in vivo* and *in vitro* experiments. They suggest that the toxin, in causing paralysis, acts mainly by decreasing acetylcholine synthesis, which results in functional defects at the myoneural junction. According to a personal communication from Dr. Carl Lamanna of The Johns Hopkins University, Dr. F. Dickens of Middlesex Hospital Medical School of London, England, using pure botulinus toxin failed to repeat the results of Torda and Wolff.

Lamanna (1948) observed that crystalline and amorphous preparations of toxin causes the agglutination of the red cells from all animal species tested (chicken, guinea pig, rabbit, sheep, man). Unlike the agglutination of the red cells by viruses, toxin does not appear to be taken up by the clumped red cells under the conditions studied. The agglutinating activity of the toxin appears to run parallel with its toxicity. This property is specifically prevented by type A antitoxin. The order of adding the reagents namely, toxin, antitoxin, and red cells does not affect the inhibition by antitoxin. The nature of the hemagglutinating property of the toxin is not as yet clearly understood.

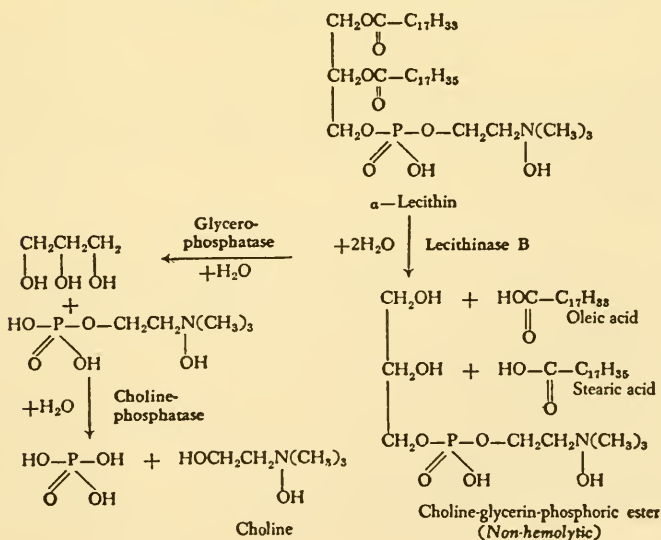
**c. Crystalline Tetanal Toxin.** Pillemer, *et al.* (1946) reported the crystallization of a toxic protein from the filtrates of *Clostridium tetani*. The crystalline toxin contained between 50,000,000 to 75,000,000 mouse minimal lethal doses (MLD)/mg. of toxin nitrogen.

One mouse MLD, or 0.000013 mg of toxin nitrogen was sufficient to kill a mouse within 96 hours with an incubation period of about 30 hours (Pillemer and Wartman, 1947). Injection of 500,000 times this amount of toxin, or 6.4 mg of toxin nitrogen, produced tetanal signs in 35 minutes and death within one hour (without producing pathological symptoms). As possible mode of action of the toxin, Pillemer and Wartman suggest that tetanal toxin itself is actually only an intermediate agent in the production of tetanus and that another substance formed in the host after the administration of tetanal toxin is the actual toxic agent. Thus, the only time which would be required for the manifestation of tetanal symptoms would be that which was necessary for the production of the actual toxic agent.

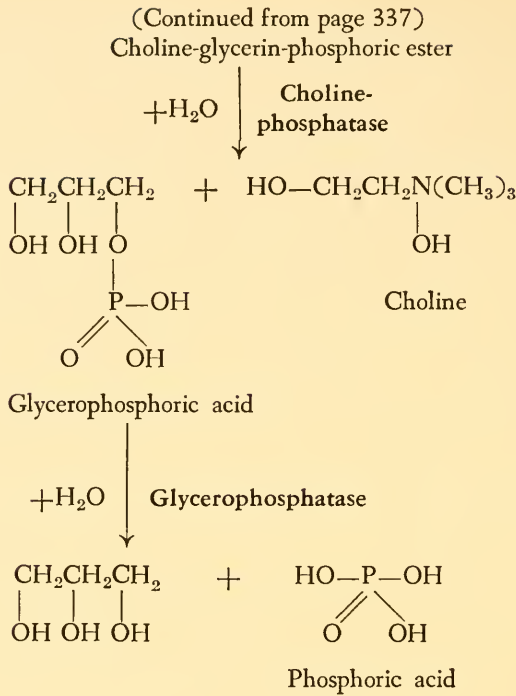
The chief clinical signs with the crystalline toxin were fixation of the muscles of the thorax and abdomen, respiratory embarrassment, increased muscular tonus and asphyxia.

d. **Enzymatic Activities of the Toxins of *Vibrio Comma*.** Felsenfeld (1944) reported that the filtrates of four *Vibrio comma* and one El Tor strain of vibrio showed lecithinase activity liberating choline, free phosphate, and unsaturated and saturated fatty acids from lecithin. These findings indicated the presence of lecithinase B, choline-phosphatase and glycerophosphatase in the filtrates. The course of reactions could be schematized in the following manner:

*Lecithinase, choline-phosphatase and glycerophosphatase of Vibria Comma*







2. Types of Toxins of *Clostridium Oedematiens*

Oakley, *et al.* (1947) found that, with the exception of *Cl. histolyticum*, the culture filtrates of *Cl. oedematiens* (*Cl. novyi*), types A and B, *Cl. haemolyticum*, *Cl. sporogenes*, *Cl. sphenoides* and *Cl. sordellii* were lecithinase positive.

TABLE XVI

Activities of Toxins	Designation of Toxins	Presence in <i>Cl. oedematiens</i>		
		Type A	Type B	Type C
Lethal, necrotizing . . . . .	<i>a</i>	+	+	—
Hemolytic, necrotizing lecithinase .	<i>β</i>	—	+	—
Hemolytic lecithinase . . . . .	<i>γ</i>	+	—	?+
Oxygen-labile haemolysin . . . . .	<i>θ</i>	+	—	—
Opalescence in lecitho-vitellin;				
? pearly layer . . . . .	<i>ε</i>	+	—	—
Hemolysin . . . . .	<i>ζ</i>	?—	+	—

After an extensive study of the properties of the toxins elaborated by 103 strains of *Cl. oedematiens*, types A, B and C, Oakley, *et al.* (1947) presented their findings in Table XVI.

Like those of *Cl. welchii*, the lecithinase activities of  $\beta$ - and  $\gamma$ -toxins of *Cl. oedematiens* were activated by calcium ion. These toxins readily attacked horse red cells, which are hardly attacked by *Cl. welchii*  $\alpha$ -toxin; sheep red cells, which are hardly affected by *Cl. welchii*  $\alpha$ -toxin, are relatively insensitive to *Cl. oedematiens*  $\beta$ - and  $\gamma$ -toxins. Evidently, something is involved in the hemolysins of these different types of red cells besides the enzymatic attack on lecithin.

They reported that whatever lecitho-vitellin values are chosen for comparison, they bear no relationship whatever to the anti-lethal values. Furthermore, they observed that *Cl. oedematiens*, capable of producing dense opalescence in lecitho-vitellin, may show no lethal activity.

The above classification of the toxins of *Cl. oedematiens* differs decidedly from that of the toxins of *Cl. welchii*. In the latter, the lethal effect of  $\alpha$ -toxin is associated not with hemolytic but with the lecithinase activity. In the absence of information concerning the hydrolytic products of lecithin as the result of the action of  $\gamma$ -hemolytic lecithinase of *Cl. oedematiens* a comparison of the pertinent enzymes of the two species of Clostridia cannot, at present, be made.

### 3. Lecithinase of *Clostridium Welchii*

a. **Classification and Properties of the Toxins of *Clostridium Welchii*.** *Cl. welchii* has been classified into four types, A, B, C and D, differing from each other serologically (Wilsdon, 1931; 1932–1933). Glenny, *et al.* (1933) differentiated the toxins of these four types into  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\theta$  and  $\epsilon$ . Since one or more of these toxins are present in the culture filtrates of a given type of *Cl. welchii*, the following table is prepared to indicate the properties of various toxins.

Prigge (1937) claimed to have shown that the toxin corresponding to  $\alpha$ -toxin of Glenny, *et al.* is non-hemolytic. He believed therefore, he had demonstrated the presence of a new toxin and called it *zeta* (' $\zeta$ ') toxin. MacFarlane, Oakley and Anderson (1941), however, pointed out that the failure of Prigge to demonstrate the hemolytic activity of this toxin is due to the use of phosphate buffer for making toxin

TABLE XVII

Activities of Toxins	Designation of Toxins	Neutralized by	Not Neutralized by	Toxins Present in Types of Clostridia			
				A <i>Cl. welchii</i>	B <i>Cl. welchii</i> agni type	C <i>Cl. paludis</i>	D* <i>Cl. ovitoxicus</i>
Lethal, hemolytic, demonecrotic and lecithinase activities Lethal and demonecrotic . . . . .	$\alpha$	$\alpha$ -antitoxin anti-serum to <i>Cl. paludis</i> toxins or lamb dysentery bacillus	—	+	+	+	+
	$\beta$		$\alpha$ -antitoxin	—	+	+	—
Lethal (to mice) . . .	$\gamma$	anti-serum (horse) to <i>Cl. paludis</i> filtrates	$\alpha$ - or $\beta$ -antitoxin	—	+	+	—
Hemolytic . . . . .	$\theta$	$\theta$ -antitoxin	Low conc'ns of $\alpha$ -antitoxin	+	—	+	—
Lethal and demonecrotic . . . . .	$\epsilon$	anti-serum to lamb dysentery bacillus	—	—	+	—	+

A=Responsible for gas gangrene in man. Also causes gas gangrene in the form of puerperal sepsis. Causes gas gangrene in animals, especially sheep. Also pathogenic to mice, pigeons, and less so to rabbits. B=Responsible for lamb dysentery. C=Responsible for "struck"—an enteritis of sheep. D=Responsible for an enterotoxemic disease and for pulpy kidney disease of sheep.

\*See footnote on page 341.

dilutions. They stated that the *zeta* toxin of Prigge is identical with the  $\alpha$ -toxin of Glenny, *et al.*

Van Heyningen (1941a) confirmed the above objection of MacFarlane, *et al.* by demonstrating that the hemolytic activity of  $\alpha$ -toxin (*zeta* of Prigge) requires the presence of calcium ion and that the presence of phosphate buffer suppresses hemolysis by  $\alpha$ -toxin. Phosphate buffer had no suppressing effect on hemolysis by  $\theta$ -toxin. Van

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*\*Comment on the So-Called Conversion of  $\epsilon$ -Prototoxin into  $\epsilon$ -Toxin of Cl. welchii Type D.* Bosworth and Glover (1934-1935) observed that the culture filtrates or solutions of dried "toxin" of *Cl. welchii* D acted upon by small quantities of trypsin became, after a short period, much more toxic. This phenomenon was prevented if the trypsin was previously treated with normal "anti-tryptic" serum, apparently due to the presence of trypsin inhibitor in the normal serum (see p. 163). Crude trypsin, purified trypsin, and a "purified form of chymotrypsin" increased the activity of crude type-D toxin, but pepsin, papain, diastase and amylase did not. Despite the increased toxicity the combining power was not changed, and they considered this as additional evidence that the results cannot be explained by the supposed presence of an inactive prototoxin which is converted into the  $\epsilon$ -toxin by trypsin.

Turner and Rodwell (1943a, 1943b) report that the cultures of most strains of *Cl. welchii* type D contain, in addition to  $\epsilon$ -toxin, an almost atoxic relatively thermostable  $\epsilon$ -prototoxin with the same combining power as  $\epsilon$ -toxin, and that activation is the result of proteolytic conversion of prototoxin into toxin. They suggest that in type-D cultures  $\epsilon$ -toxin develops from prototoxin through the proteolytic action of intrinsic protease. Their conclusions are listed here: (a) a precursor of  $\epsilon$ -toxin is excreted from the cells during the growth phase; (b) this precursor is atoxic or relatively so; (c) it is more thermostable than  $\epsilon$ -toxin; (d) it has the same capacity to combine with specific globulin as toxin has; (e) it is equally antigenic; (f) the groups to which it owes its toxicity are "masked" by something that can be digested away by a variety of proteases, including type-D protease; and (g)  $\epsilon$ -toxin is derived from  $\epsilon$ -prototoxin and is not excreted directly by the cells.

It is to be noted that the  $\epsilon$ -prototoxin is antigenically complete but is atoxic. Its power to combine with the antitoxin is the same as that of fully active toxin. During the growth of the organism maximal combining power and potential toxicity are reached in cultures after only a few hours' growth, whereas primary toxicity may not become maximal for four or more days. The above observations show that the complete toxin molecule is formed in its antigenically specific and potentially toxic form, and, most likely, secreted from the cells in combination with another non-toxic protein in the form of an atoxic molecular complex. It does not appear reasonable to assume that such a complex conforms with the precursor idea. Since the maximal combining power and the potential toxicity are reached in cultures after only a few hours' growth, it is reasonable to consider that these properties are inherent in the free toxin molecule or the atoxic molecular complex. The proteolysis of the atoxic complex eliminates the non-toxic protein from the atoxic complex, exposing the toxic groups already present in the original toxin molecule. In another section of this monograph, several examples are given showing clearly how indifferent proteins combining with active viruses and enzymes form inactive and readily dissociable molecular complexes. It would seem that Turner and Rodwell's  $\epsilon$ -prototoxin possesses features similar to such protein-protein complexes. These considerations do not lend support to the supposition that the atoxic complex is the primary product in the chemical genesis of the fully developed toxin molecule.

Heyningen separated quantitatively  $\alpha$ - from  $\theta$ -toxin present in a culture filtrate by adsorption of the latter toxin on red blood cells. The  $\alpha$ -toxin content of the supernatant, as measured by the turbidometric method (van Heyningen, 1941b), was unchanged, or slightly diminished, but the hemolytic activity when measured in saline containing calcium ion, was high. When the toxic filtrate was left with the red cells for longer than five minutes, more  $\alpha$ -toxin was adsorbed, but the adsorption of the  $\alpha$ -toxin did not begin until all the  $\theta$ -toxin had been adsorbed.

The hemolytic activity of the  $\theta$ -free preparations was completely inhibited by low concentrations of Glenny's  $\alpha$ -antitoxin and unaffected by high concentrations of his  $\theta$ -antitoxin.

**b. Preparation of Toxins.** MacFarlane and Knight (1941) used the following method. The medium on which *Cl. welchii* was grown was composed of 3.3 per cent peptone (800 ml.),  $\text{Na}_2\text{SO}_4$  muscle-extract (100 ml.) from horse and beef and 0.5 per cent glucose. It was sterilized by filtration and adjusted to pH 7.6. Incubation for five to six hours produced a maximum toxin concentration.

Several preparations of dried toxin were made by 2/3 or full saturation of the filtrate with ammonium sulfate. The average yield was 1 g. per l. of medium. The M.L.D. of the toxin preparations used in this study were as follows: Dried toxin—0.008 mg.; glycerinated toxin—0.0025 mg.\*

**c. The Lecithinase Activity of the Toxin.** The activity of the diluted toxin on lecithin was followed by allowing it to act on egg yolk under standard conditions. The reaction was stopped by addition of excess antitoxin and the turbidity measured in a colorimeter against that developed simultaneously by a known amount of toxin under the same conditions. The total trichloroacetic acid soluble phosphorus in the reaction mixtures was determined by preliminary ashing with perhydrol and  $\text{H}_2\text{SO}_4$ , and colorimetric estimation of the inorganic phosphorus. The results showed that by the action of the toxin the original lipid phosphorus was converted into a trichloroacetic acid-soluble form. Most of the phosphorus originally present in the protein component also became acid-soluble, although the protein nitrogen was only slightly decreased. A comparison of the relative degree of hydrolysis and of turbidity showed that at pH 7.1 and 5.9 the rate of

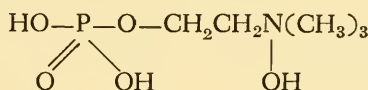
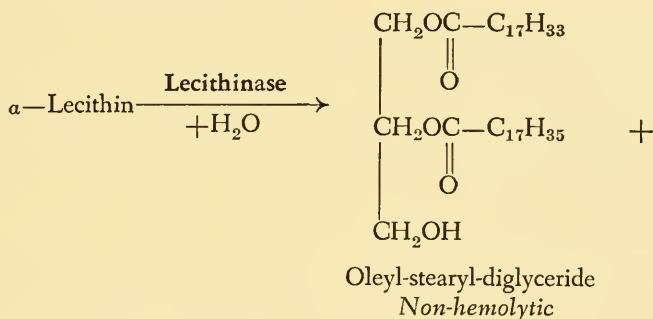
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\*Logan *et al.* (1945) described growth conditions of *Cl. welchii* capable of producing 800–1000 L.D.50/ml. of type A  $\alpha$ -toxin.



hydrolysis and the development of turbidity were almost parallel, while at pH 9.3 the hydrolysis was considerably faster than the increase in turbidity. The latter was explained by assuming that the hydrolysis of the phospholipid is the primary action, and that the aggregation of fat globules to which the turbidity is due is dependent on this hydrolysis but influenced by the physical conditions of the reaction mixture.

Following the course of the hydrolysis of lecithin by toxin and identifying the reaction products, they found that the aqueous solution of the hydrolysate contained practically all of the original lipid phosphorus in an organic form, and an equivalent amount of nitrogen, but no free choline. The hydrolytic products were characterized as phosphorylcholine and a diglyceride.

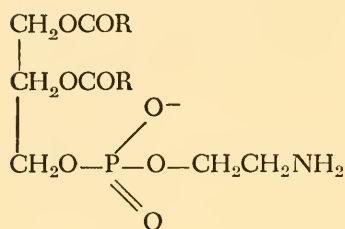
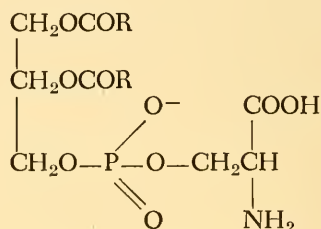


Choline-phosphoric acid

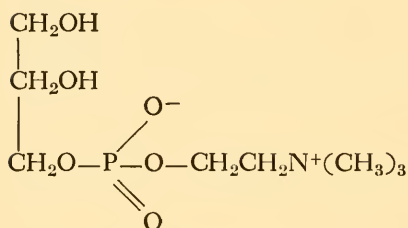
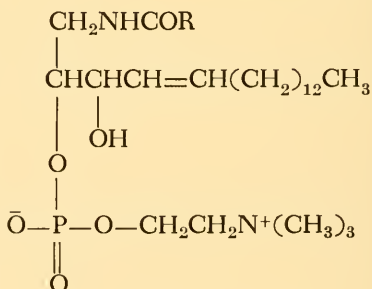
Zamecnik, Brewster and Lipmann (1947) reported that *Cl. welchii* lecithinase is completely inactive against phosphatidylserine, sphingomyelin\*, ox brain cerebroside, glycerophosphoryl choline and soy bean

\*According to MacFarlane (1948) the toxic culture filtrates of *Cl. welchii* contain a lecithinase which is probably identical with the main lethal component of the filtrates, the  $\alpha$ -toxin. This lecithinase did not hydrolyze cephalin but did hydrolyze slowly *sphingomyelin*. The *Cl. welchii* toxins were observed to manifest the peculiar specificity in *splitting off phosphorylcholine from lecithin and sphingomyelin* but failing to attack cephalin or *glycerophosphorylcholine* and *lysolecithin*.

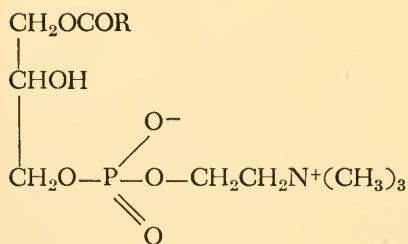
phosphatides. They also reported that a 2.5 per cent solution of oleyl-stearyl-diglyceride, a 2 per cent solution of phosphoryl choline, 0.06 per cent solution of glycerophosphoryl choline do not inhibit lecithinase (2.5 per cent concentration). In 0.7 per cent concentration, the related phosphatidyl serine and phosphatidyl ethanolamine are inhibitory. So also, ox brain cerebroside, sphingomyelin, and a sorbitol-mannitol oleic acid ester (Tween 30) are inhibitory. Part of the inhibitory effect of phosphatidyl serine was traced to the removal of

 $\alpha$ -Phosphatidyl ethanolamine

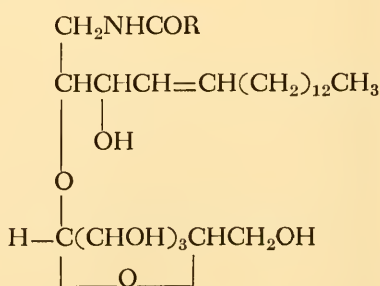
Phosphatidyl serine

 $\alpha$ -Glycerophosphoryl choline

Sphingomyelin



Lysolecithin



Cerebroside

calcium ion, essential for the lecithinase reaction, from the field of reaction due to the formation of Ca-phosphatidyl serine. The structural relationships of lecithin to the above substances are shown on page 344.

**d. Characteristics of Lecithinase.** Lecithinase activity as described above was estimated (MacFarlane and Knight, 1941) by the rate of formation of acid-soluble phosphorus from an aqueous emulsion of lecithin, by the glycerinated toxin. Under the conditions of the experiment no hydrolysis of lecithin took place in the absence of toxin at pH 7.1 and 37°C. or in trichloroacetic acid at room temperature for 30 minutes. None of the toxins examined hydrolyzed diphenyl-, monophenyl-, glycerophosphate or nucleic acid; the enzyme is therefore a lecithinase.

The dilute enzyme is very rapidly inactivated by the exposure of a shallow layer, by shaking or by bubbling air through it. This inactivation was assumed to be due to surface denaturation and not to oxidation, for the enzyme was unaffected by treatment with 0.3 per cent hydrogen peroxide solution, while bubbling with purified nitrogen (passing through alkaline  $\text{Na}_2\text{S}_2\text{O}_4$ ) inactivated it. The percentage of inactivation of toxin was determined by animal test and by its action on egg-yolk.

Toxin lecithinase was found to be comparatively heat-stable, 45 per cent of the activity remaining after heating in borate buffer of pH 7.6 for 10 minutes at 100° in a sealed ampoule. It was more rapidly inactivated in acid than in alkaline solution at this temperature. The enzyme was active over a wide pH range of 7.0 to 7.6, in borate buffer; the activities at pH 9.3 and 5.2 in borate and acetate buffers were, respectively, 64 and 66 per cent of the activity at optimal pH. The activity of the enzyme was greatly affected by the presence of calcium ions.

Low concentrations of magnesium salts also activated, but higher concentrations of 0.01 M or more inhibited the enzyme.

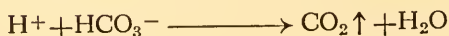
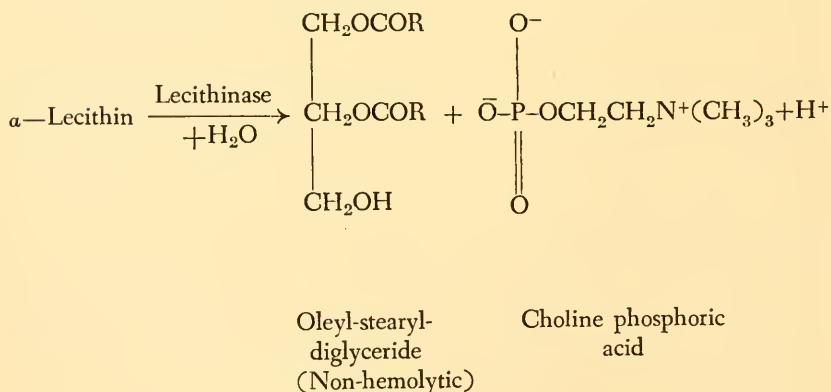
Toxoids had no lecithinase activity, nor did they influence the lecithinase activity of toxins. The lecithinase activity of traces of  $\alpha$ -toxin present in dry type C toxin was not inhibited by the presence of  $\beta$ - and  $\delta$ -toxins in type C toxin.

**e. Quantitative Methods Used for the Measurement of Lecithinase Activity: Acid-Soluble Phosphorus Method.** MacFarlane and Knight (1941) measured the rate of formation of acid-soluble

phosphorus from an aqueous emulsion of lecithin as a measure of lecithinase activity. A lecithinase unit, arbitrarily defined, was that quantity of enzyme which under the conditions prescribed above produced 0.1 ml. of acid-soluble phosphorus from lecithin in fifteen minutes at 37°C. The relationship between the minimum lethal dose, the egg-yolk unitage and the lecithinase activity of toxins, respectively, of a pool of toxins was 400, 200, and 210. Theriault (1945) carried out a detailed study of the factors insuring the optimal potency of the  $\alpha$ -toxin based on lecithinase activity. His specifications differ from those proposed by MacFarlane and Knight chiefly in the use of more lecithinase and of more buffer, also in the use of a higher temperature (45°C.) incubation.

*Turbidometric Method.* Lecithinase produces opalescence in some samples of human serum both from normal and diseased persons. A clear filtrate of lecitho-vitellin prepared by heating a saline suspension of egg-yolk and filtering through a Seitz-filter has also been used as substrate for this reaction. Following the detailed study of opalescence by MacFarlane, Oakley and Anderson (1941), van Heyningen

Lecithinase ( $\alpha$ -Toxin) of *Cl. welchii*



(1941b) developed a refined technique to determine the concentration of  $\alpha$ -toxin in solutions by measurement of the turbidity developed.

*Manometric Method.* A manometric method for the measurement of lecithinase activity has been worked out by Zamecnik, Brewster and Lipmann (1947). Lecithinase catalyzes the hydrolysis of lecithin into phosphocholine and oleyl-stearyl-diglyceride. The hydrogen ion from the former reacts with bicarbonate ion liberating carbon dioxide into the gas phase. Electrometric titration of phosphocholine revealed a  $pK_2$  of 5.6. In a bicarbonate-carbon dioxide buffer system of pH 6.74, approximately 95 per cent of the previously formed choline phosphoric acid immediately reacted with bicarbonate ion, liberating carbon dioxide into the gas phase. In a comparison between the acid-soluble phosphorus method and the manometric method, a correction for the latter method, amounting to not more than 10 per cent, was indicated.

**f. Phenomenon of Opalescence in Serum and Lecitho-Vitellin Produced by the Action of the Toxin of *Cl. Welchii*.** Seiffert (1939) and Nagler (1939) reported that toxin of *Cl. welchii* (*Cl. perfringens*) was capable of producing an obvious opalescence in some samples of human serum both from normal and diseased persons. No effect was observed with toxoid. A reinvestigation of this phenomenon by MacFarlane, Oakley and Anderson (1941) resulted in the following findings. They found that when *Cl. welchii* toxin was mixed with some human sera a very marked opalescence due to fat was observed within a few hours. A clear filtrate of lecitho-vitellin prepared by heating a saline suspension of egg-yolk and filtering through a Seitz-filter was found to be extremely stable so long as it remained sterile. When this crude lecitho-vitellin solution was treated with *Cl. welchii* toxin, even in the cold, opalescence appeared within a few minutes, proceeding to flocculation and separation of a thick curd of fat. If lecitho-vitellin is extracted with ether and the ether-extracted material is then treated with the toxin, a much reduced opalescence occurred. The extraction of the opalescent mixture with ether yielded a fat.

The greater part of the opalescence produced in crude lecitho-vitellin was due to the aggregation of finely divided free fat, a small part of which was stated to be due to the setting free of fat from compounds, probably lipo-proteins, as some proteins were denatured at the same time.



$\beta$ ,  $\delta$ ,  $\theta$  and  $\epsilon$ -toxins were found to exercise no effect upon human serum or lecitho-vitellin, and antitoxins to group A, B, C and D, if containing insufficient antitoxin to neutralize the  $\alpha$ -toxin as judged by the hemolytic or lethal testing, failed to prevent the reaction of toxin with serum and with lecitho-vitellin. It thus appears that the active principle in toxins causing the phenomenon of opalescence, etc., in human sera, is contained in  $\alpha$ -toxin and is neutralizable only by  $\alpha$ -antitoxin.

Potassium oxalate, disodium phosphate and sodium citrate prevented both hemolysis and opalescence, suggesting that ionized calcium, which was found by MacFarlane and Knight (1941) to be an accelerating factor of lecithinase activity, might play an essential part in the reaction. The restoration of calcium in the system removed by the above salts, led to the restitution of all of the *in vitro* activities of the toxin. Thus it is clearly shown that hemolysis by  $\alpha$ -toxin and its reaction with serum and lecitho-vitellin, as described above, occur only in the presence of ionized calcium.

The above discussed phenomenon of opalescence produced by the action of the  $\alpha$ -toxin of *Cl. welchii* on sera and lecitho-vitellin appears to be identical with that observed in the experiments of MacFarlane and Knight (1941) in which the action of  $\alpha$ -toxin on clear egg-yolk produced a turbidity which increased as the hydrolysis of lecithin progressed. Both studies show clearly that this toxin exercises a powerful hydrolytic action on lecithin and lecitho-vitellin that not only splits lecithin into a diglyceride and phosphocholine, but also causes the splitting of lecithin from lecitho-vitellin and the "denaturation" of the protein component. The splitting of lecithin from its conjugation with the protein (serum or egg-yolk) appears to be due to a primary effect of "denaturation" or "proteolysis" on the protein component by toxin. Such an effect by toxin will naturally result in the rupture of the bonds between lecithin and the protein in a manner similar to the splitting of the prosthetic groups of conjugated proteins by denaturation or proteolysis of the protein components (Sevag and Smolens, 1941). The hydrolysis of lecithin by the lipase activity of toxin would represent, therefore, a second stage in the occurrence of the phenomenon of opalescence. The above assumption that toxin exercises a primary effect of "denaturation" or proteolysis on the conjugated lipo-protein or lecitho-vitellin of serum would seem to gain a certain degree of

support as discussed below from the findings of Maschmann (1937) that the toxin of *Cl. welchii* (*Cl. perfringens*) also exercises a strong proteolytic activity (p. 256).

**g. Production of Opalescence in Serum and Lecitho-Vitellin as a Measure of the Potency of the Toxin.** As discussed previously, Seiffert and Nagler observed that the toxin of *Cl. welchii* produces an obvious opalescence in some samples of human serum. This effect was also produced by the action of toxin on lecitho-vitellin. It is evident that enzymatic action of the toxin produces definite chemical changes in the constitution of the above mentioned lipo-proteins. This phenomenon has been studied in some detail, as discussed above, by MacFarlane, *et al.* They observed that any process which reduced the hemolytic activity of the toxin also reduced its capacity to produce opalescence in serum and lecitho-vitellin. This strict parallelism between hemolysis by  $\alpha$ -toxin and the production of free lipoid from lecitho-vitellin—a known lipo-protein—led these authors to suppose that these activities might be manifestations of the same enzyme reaction, since hemolysis may well follow the breakdown of the lipo-protein complex in the red cell envelope.

As the hemolytic activity of the toxin was neutralized by the specific antitoxin, so was the enzymatic activity of the toxin, responsible for the production of opalescence in lipo-proteins, neutralized by the same antitoxin. The potency of the antitoxin in producing these two effects ran parallel. Neither the hemolytic activity nor the production of opalescence were neutralized by antitoxins prepared against other toxins. These facts showed a high degree of identical specific action characteristic for both the enzyme and antitoxin reactions.

The hemolysis and the production of opalescence by  $\alpha$ -toxin were found to be dependent on the presence of calcium ions. In the absence of calcium, both reactions were absent. Potassium oxalate, disodium phosphate and sodium citrate, which form non-ionizable salts with calcium, prevented both hemolysis and opalescence, suggesting that ionized calcium, which was found by MacFarlane and Knight (1941) to be an accelerating factor of lecithinase activity, might play an essential part in the reaction. The restoration of calcium to the system led to the restitution of all of the *in vitro* activities of the toxin.

Another point which requires emphasis here is the relation of the proteolytic activity of the toxin to the phenomenon of opalescence.

It was suggested above that the proteolytic activity of the toxin might split or hydrolyze protein prior to the occurrence of the phenomenon of opalescence. As found by Maschmann (1937) antitoxin neutralizes the proteolytic activity, which effect might also be related to the inhibition of opalescence (hemolysis as well) by antitoxic sera.

Following the detailed study of the phenomenon of opalescence by MacFarlane, Oakley and Anderson (1941), van Heyningen (1941) developed a refined technique to determine the concentration of  $\alpha$ -toxin in solutions by the measurement of the turbidity developed. He presented this method as a more accurate and economical method of measuring the lethal, dermonecrotic and hemolytic properties of toxin. He stated that the specific estimations of  $\alpha$ -toxin by these three methods are open to various objections, besides the time consumed and the expense involved in carrying out the mouse experiments.

The substrate for toxin-enzyme action was prepared from the yolk of a day-old egg. The turbidity developed was measured colorimetrically. When amounts of toxin corresponding to 1-6 M.L.D. were incubated for 15 minutes at 38° with 2 ml. of substrate solution, fairly dense turbidities were produced, which fell outside the range of a nephelometer, but which could be conveniently measured in an ordinary colorimeter. The degree of turbidity developed in a given time was taken as a measure of the amount of toxin present. To stop the reaction at the end of a desired period he added an equivalent amount of the isotonic saline solution containing 2-3 antitoxin units per ml. The activity of several samples of toxin could thus be determined within an hour, with very satisfactory accuracy, and without resorting to a large number of serial dilutions. It was stated to be particularly useful in studying the toxin-antitoxin reaction since only one tube need be used in the determination of the residual activity in a toxin-antitoxin mixture.\*

**h. Lecithinase Activity as a Measure of Biological Effects of Toxin.** MacFarlane and Knight (1941) found that the lecithinase activity of the  $\alpha$ -toxin is completely inhibited by the specific antitoxic

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\*Crook (1942) reported the results of his study of the Seiffert-Nagler reaction (production of opalescence) with forty batches of toxin from thirteen different type A strains and one type D strain of *Cl. welchii*. He found that the correlation between end point (Seiffert-Nagler reaction) and mouse M.L.D. was remarkably good through-

serum. This inhibition was carried out as follows: Two ml. of diluted toxin (1 to 2 lecithinase units) were allowed to react with a saline dilution of the serum in a total volume of 3.8 ml. for 15 minutes at room temperature; 0.2 ml. of 0.3 M  $\text{CaCl}_2$ , 1 ml. of borate buffer of pH 7.1 and 1 ml. of 2.5 per cent lecithin were added and the amount of hydrolysis in 15 minutes at  $37^\circ$  was estimated. A control without the immune serum was likewise included.

The results of numerous experiments showed that the hydrolysis of lecithin by 1.9 lecithinase units of toxin was inhibited from 13 to 95 per cent by amounts of antiserum containing from 0.01 to 0.1 antitoxic units. The lecithinase activity was not significantly altered by the addition of normal or diphtheria antitoxic horse sera in quantities 10 to 50 times greater than those of the *Cl. welchii* antitoxic sera used.

When the lecithinase activity was measured in the presence of calcium ion, a larger amount of antitoxin was required for the 100 per cent neutralization of a given amount of toxin than in the absence of calcium. To obtain complete neutralization of a given amount of toxin, for example, it required only 0.1 unit of antitoxin in the absence of, and 0.19 unit in the presence of calcium ion. The effect of calcium on the minimum hemolytic dose was similar. In the absence of calcium, the minimum hemolytic dose of toxin was about three times as much as in its presence. In this connection it is also interesting to note that the toxoid, which is devoid of characteristic toxin activities was likewise found to have lost the lecithinase activity. Unlike the snake venom lecithinase which produced hemolytic lysolecithin, the lecithinase of the  $\alpha$ -toxin of *Cl. welchii* hydrolyzes lecithin into non-

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out. The correlation was even more accurate when the photo-electric method was used. *Cl. oedematiens* strains produced reactions as strong as *Cl. welchii* filtrates made under similar conditions. Crook stated that species such as *Cl. sporogenes* and *Cl. tertium* should be included among positives. In all cases where they were available, the homologous antitoxins completely inhibited the reactions. Heterologous sera did not neutralize the toxins in the Seiffert-Nagler reaction. His results also showed that the reaction is due to the  $\alpha$ -toxin of Glenny. The lipid material separating during the reaction is complex and consists of various types of lipoids and protein.

The kinetics of the reaction in serum and egg-saline are characterized by a pronounced induction period, and are typical of an enzyme or enzymes.

Kass, *et al.* (1945) studied 94 strains of *Cl. welchii* isolated from human and animal feces and soil with respect to the interrelation of virulence and lecithinase (and hyaluronidase) production. Seventy per cent of all the strains produced lecithinase. Of the 46 per cent virulent strains only 17 per cent failed to produce lecithinase. In a review, Reed (1943) discussed various aspects of clostridia responsible for gas gangrene.



hemolytic phosphocholine and a diglyceride (stearyloleylglyceride). These facts appear to show that the decomposition of lecithin by *Cl. welchii* toxin constitutes a more drastic change in lecithin, since the phosphoric acid group is now present as a water-soluble compound, but the products of the decomposition are stated to be innocuous. On the other hand, considering the activity of toxin from the pathological point of view these authors expressed the view that the lecithinase of *Cl. welchii* toxin is probably identical with the lethal, hemolytic and necrotic factors of this toxin. This appeared to be evident since the degree of inhibition of the lecithinase by various type A antisera ran parallel with the protective power of the sera *in vivo*. In two type C sera, the antilecithinase activity was in good agreement with an independent assay of the  $\alpha$ -antitoxin content. Although the presented evidence strongly suggested that the lecithinase and  $\alpha$ -toxin are identical, the ultimate proof of this was held to be dependent on the isolation of homogeneous toxin with the requisite biochemical and pathogenic properties. The lecithinase activity of the toxin examined was found to be so high as to be capable of accounting for the pathological effect; an amount roughly equivalent to 1 M.L.D. for a mouse could at its maximum velocity hydrolyze the whole of the blood lecithin of the animal in two to three hours.

#### 4. Immunity to the Pharmacological Actions of Toxins

The enzymatic activities of the various toxins discussed above are more or less directly related to their biological effects. In the first place it has been shown that the neutralization of toxin by antitoxin inhibits the enzymatic activities of the toxins. Secondly, the pharmacological effects of the toxins are likewise related to one or more enzyme activities of the toxins.

a. **The Inhibition by *Cl. Welchii* Toxin of the Oxidation of Succinate by Tissue.** In the following pages it will be shown that toxins cause the liberation in animal organs of various substances injurious to tissue. The tissue freshly obtained from an animal is capable of oxidizing certain enzyme substrates. These respiratory enzymes are susceptible to various specific and non-specific enzyme poisons. Wooldridge and Higginbottom (1938) found that the toxins of *Cl. welchii* inhibit the oxidation of succinate by aqueous suspensions of minced small



intestine of guinea pig and that this inhibitory effect by toxin is neutralized by antitoxic sera.

The suspension of the minced small intestine of a guinea pig oxidized succinate, p-phenylenediamine and dihydroxyphenylenediamine. The following substances were found to be non-oxidizable by the enzyme suspension: glucose, fructose, sucrose, lactate, pyruvate, fumarate, malate, oxalate, butyrate, oxalacetate, citrate, formate, glycerol, alanine, serine, tryptophane, glutamate, aspartate, phenylalanine, glycylglycine, leucylglycine, glycyltryptophane or glycyltyrosine.

The toxins of *Cl. oedematiens*, *Cl. septicum*, *Cl. tetani*, and *Cl. welchii*, types A, B, C, and D were similarly tested. Of these, only the toxins of *Cl. welchii* produced inhibitions significantly greater than that of the broth precipitate, and of these inhibitions, that induced by the type A organism was the greatest and that by the type D the least. The extent of the inhibition varied with the amount of toxin added. At the concentration of 0.4 mg. of toxin per system, the inhibition by the toxins of the A, C, D types of organisms were respectively 84.21, 64.01 and 29.48 per cent.  $\alpha$ -Toxin was present in all of these three toxins. The  $\beta$ -toxin was only present in the toxin of type C organism (100 toxin units per 100 mg.) and the  $\epsilon$ -toxin only in that from type D (800 units of toxin/100 mg.) and, in comparison with  $\alpha$ -toxin, their concentrations were high. On the basis of comparative studies, the authors suggested that the inhibition of the succinate oxidation may be due to the  $\alpha$ -toxin, but that, in any case the inhibitory actions of the  $\beta$ - and  $\epsilon$ -toxins must be small.

The toxin of *Cl. welchii* caused a 39 per cent inhibition of the oxidation of dihydroxyphenylalanine, and 27.42 and 84.32 per cent inhibition, respectively, of phenylenediamine and succinate. These are known to be specific substrates for the cytochrome-cytochrome-oxidase (indophenoloxidase) respiratory systems.

The oxidation of succinate by other tissues was studied and it was found that the toxin of type A *Cl. welchii* caused 21.66 to 74.0 per cent inhibition of the succinate oxidation by skeletal and heart muscles, liver, kidney and brain tissues. A dried preparation of 'lactic dehydrogenase,' which oxidized succinate much more actively than lactate or malate, was also inhibited about 80 per cent by various toxins. In contrast, in experiments of a preliminary nature, no inhibitory effect

by the toxins on the oxidation of lactate or malate by this enzyme preparation was observed. The anaerobic reduction of methylene blue in the systems containing minced intestine or dried enzyme preparation and succinate was not inhibited by the toxins. Furthermore the presence of 1:3000 of methylene blue, acting as an "oxygen carrier," reduced the inhibition of aerobic oxidation of succinate by toxin from 78 to 44 per cent.

The minced small intestine gave a very strong positive reaction for indophenol oxidase, although it was practically unaffected by the addition of the toxin of *Cl. welchii*, type A, but was inhibited by cyanide which is a specific heme-enzyme inhibitor. On the basis of these findings the authors stated that "These results, together with the aerobic oxidation of succinate by small intestine in the presence of toxin and methylene blue suggest that the inhibitory action of the toxin is not exerted directly either upon the dehydrogenase or upon the oxidase but may be associated with an interference to some intermediate link, possibly with an inactivation of some 'carrier catalyst.'" They emphasized the fact that such interference by bacterial toxins might facilitate the subsequent invasion of the body by the toxigenic organism itself or by some other organism present at the time. This was compared with the absence of certain vitamins, e.g., lactoflavin or ascorbic acid, associated with the respiratory mechanisms of cells, leading to grave disease.

**b. Neutralization of the Inhibition of Tissue Respiration by Toxin as a Measure of the Antitoxin Concentration of Immune Serum.** Wooldridge and Higginbottom (1938), as discussed above, showed that  $\alpha$ -toxin of *Cl. welchii* inhibited the oxidation of succinate by tissue. This inhibition was neutralized completely by specific antitoxin.

Of the antisera used, only those for the three types of *Cl. welchii*, A, C, and D, contained antibodies for the  $\alpha$ -toxin. The type A antitoxic serum contained 300 units of  $\alpha$ -antitoxin per ml.; type C antiserum contained 120 units of  $\alpha$ -antitoxin and 900 units of  $\beta$ -antitoxin per ml.; and type D antiserum contained 85 units of  $\alpha$ -antitoxin and 275 units of the  $\epsilon$ -antitoxin per ml. When these three antitoxins were added in amounts equivalent in  $\alpha$ -antitoxin units, they each reduced the inhibitory effect of the type A toxin by about half, as shown in the following table.

TABLE XVIII

Antiserum	$\alpha$ -Antitoxin units/ml.	Dilution	Neutralization of inhibition
			<i>Per cent</i>
Type A	300	1:10,000	51.7
Type C	120	1:4,000	44.7
Type D	85	1:2,800	59.3

Moreover, the degree of neutralization of the inhibition was proportional to the  $\alpha$ -antitoxin concentration; an antitoxin concentration of  $4 \times 10^{-4}$  produced 88 per cent neutralization. Other antisera, such as septicæ, oedematis, tetanus, and normal serum produced no appreciable inactivation of the toxin.

c. **Pharmacological Activity of the Toxins of *Cl. Welchii*.** Biological changes resulting from the action of toxins has been the subject of a series of studies by Kellaway, Trethewie, and Turner (1940a, 1940b) and Kellaway and Trethewie (1941). The following discussion is based on their findings.

In the first two studies, they investigated the effects of the toxin of *Cl. welchii* of type D. The culture filtrate was treated with ammonium sulfate to obtain 65 per cent saturation. The precipitated toxin was then treated with 50 per cent alcohol in the cold to remove the histamine-like impurities, and further purifications were effected in the cold. The M.L.D. of the final product for a 20 g. mouse was 8 micrograms, or  $8 \times 10^{-6}$  g. The symptoms fairly regularly observed by intravenous injections in young lambs were similar to the clinical manifestations of poisoning and particularly to the convulsions which occur in the natural disease in which the toxin is absorbed from the alimentary tract. Intravenous injection in lambs caused convulsions by direct action of the toxin on the central nervous system—probably mainly upon the basal ganglia. It was stated that death occurring as a result of injections might be due to failure of respiration, or to cardiac failure. Hemorrhages in the heart in lambs and hemorrhagic edema of the lungs in lambs, rabbits and cats after intravenous injection of toxin were frequent occurrences. Antitoxin neutralized these effects of the toxin.

The authors interpreted the occurrence of hemorrhagic effects of

the toxin as responsible for the injury to tissues and thereby the liberation of histamine.\* Experiments upon the perfused lungs of cats showed the liberation of histamine from these organs by toxin, though it was much less active than snake venom. Impurities were found not to be responsible for this effect because overneutralized purified toxin failed to cause any detectable output of histamine in the perfusate. A parallelism between the degree of tissue injury and the amount of histamine liberated was established. They stated that it is probable that the action of toxin at the site of histamine liberation in the lungs contributes to the edema which is so frequently observed in all of the three species studied. It may also contribute to the liberation of adrenalin from the suprarenals of the rabbit.

As with snake venom, Kellaway and Trethewie observed that the toxin liberated adenylyl compounds. The cardio-depressant substances set free by the toxin behaved like adenosine and adenylic acid, being destroyed by the enzyme present in the tissues and in perfusates. These facts showed that toxin directly or indirectly sets in motion in the organism an enzyme or enzymes attacking nucleoproteins of tissues. To trace the cardio-depressant effect to the nucleic acid derivatives, the effects of choline derivatives in extracts and perfusates were eliminated by repeated atropinization of the test preparation. The effects of toxin in the perfusate and extracts were eliminated by overneutralization of each sample with a powerful antitoxin, and the effects of impurities in the over-neutralized mixtures were avoided by the use of doses which contained too large an amount of toxin.

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\*In connection with the nature and types of factors injurious to cells, the observations of Menkin (1943) are of interest. He has described an injury factor, tentatively called "necrosin," which is not histamine. It is present in the inflammatory exudates of dogs and man, and is associated or identical with the euglobulin fraction. It is thermolabile and non-diffusible. The dialyzed euglobulin fraction of exudates causes severe edematous inflammation in rabbits characterized by lymphatic blockade. Injected subcutaneously, it produces intense local redness, edema and frequent central necrosis, and erythema and congestion in the tributary lymphatic nodes. Intravenous administration of necrosin in a dog is followed by a marked leukopenia accompanied by transient toxic manifestations such as vomiting and diarrhea. It markedly hastens the rate of coagulation of blood *in vitro*. The question as to whether this is due to thrombokinase associated with necrosin in the latter's present state of purification is unanswered at present.

Sera containing large quantities of hemolyzed material, or highly lipemic sera, are apt to contain necrosin. It can be extracted from the blood serum of an animal which has a concomitant acute inflammation. It is absent in the euglobulin fraction of the blood serum of normal dogs and man. These facts suggested to Menkin that necrosin is liberated from injured cells.



In the third study (Kellaway and Trethewie, 1941), they reported their findings on Types B and C toxins of *Cl. welchii*. The M.L.D. of type B toxin for 20 g. mice was 0.04 mg. It was non-hemolytic and therefore probably contained no  $\alpha$ -toxin. The M.L.D. of type C toxin for 20 g. mice was 0.007 mg. The antitoxin used neutralized 16,000 to 32,000 mouse M.L.D. per ml. Since antitoxin against type C toxin caused severe circulatory effects in cats and the contraction of the jejunum and uterus of guinea pigs, particularly when mixed with toxin, it was purified by precipitating the globulin fraction, which eliminated the toxic effects.

The toxin of *Cl. welchii* type D set free both histamine and adenylyl compounds, while type A liberated adenylyl compounds but not histamine from perfused organs. The toxins of all the four types liberated adenylyl compounds from the isolated perfused liver of the rabbit. Though the preparation of type B toxin liberated histamine, it was ascribed to the presence of the  $\epsilon$ -fraction. Both toxins B and C caused liberation from the perfused liver of the rabbit, of pigment, of adenylyl compounds and of a heat labile agent which inactivates these latter compounds. Both toxins caused the contraction of the isolated jejunum of the guinea pig and of the uteri of the virgin guinea pig and rat. These responses were shown to be specific except that of the isolated uterus of the guinea pig to type B toxin—in which a histamine-like impurity was present.

## 5. Action of Antitoxin on Enzymes Causing Histological Changes in Gas-Gangrene

Robb-Smith (1945) compared the histological changes occurring in muscle lesions in gas-gangrene in man with those occurring in human muscle *in vitro* as the result of the action of *Cl. welchii* type A filtrate. It was found that the changes occurring under these two conditions were identical. These changes were considered to be due to effects by the enzymes in the filtrates on cell-membranes, cell-nuclei, collagen and reticulin, and mucoproteins, and that the myofibrils were unaffected. Antitoxin inhibited these reactions, suggesting that the local injection of antitoxin might be used advantageously where it has proved impossible to perform an adequate surgical excision of the affected tissue. However, on the basis of an extensive study of the rate



of the disappearance of antitoxin, MacLennan and MacFarlane (1945) concluded that the circulating antitoxin is incapable of arresting the local spread of gas-gangrene, and that circulating antitoxin will not prevent death, apparently from toxemia, as a result of this local infection. The presence of an excess of antitoxin in the blood-stream and wound area will not of itself prevent a fatal outcome. It was assumed that, if death is directly due to toxin and cannot be averted by antitoxin, vital tissues have been irreparably damaged by toxin before antitoxin is given, or that the antitoxin cannot protect them from circulating toxin.\* MacFarlane and MacLennan (1945) are, nevertheless, of the opinion that, while the primary lethal factor is not neutralized by antitoxin and can only be eliminated by surgery, there also is some absorption of bacterial toxin amenable to antitoxin treatment. In connection with the questions of toxin-antitoxin reaction in gas-gangrene, the observations of Zamecnik and Lipmann (1947) seem to be of considerable significance. They found that toxin-antitoxin combination is strongly inhibited if toxin and the specific substrate lecithin come together before antitoxin has a chance to react with the toxin. Even with a 20-fold excess amount of antitoxin the enzymic activity of toxin was marked. These facts would appear to indicate that in gas-gangrene, the toxin excreted by the infective agent will react with the substrate present in the tissue. Subsequent administration of antitoxin will be incapable to prevent the enzyme reaction from continuing.

In gas-gangrene toxemia, MacFarlane and MacLennan (1945) could not find lecithinase in the circulating blood, nor intravascular hemolysis in the living subject as might have been expected in the presence of hemolytic toxin. Examining gangrenous muscle they reported that the normal toughness and elasticity had been lost. It could readily be crushed between the fingers or smeared out on a plate. Microscopically recognizable muscle-fibres had fallen apart, tending to break-up into short lengths. A considerable amount of fat

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\*On the other hand, an alternative possibility is suggested by the observations, and discussions of Butler (1945). The *Cl. welchii* strains causing severe gas gangrene in men may have invasive properties unassociated with toxin production but connected with the bacterial cell. If such is the case, and since Butler finds large capsules associated with those strains causing severe infections, then perhaps for complete therapy of gas-gangrene antibacterial passive immunity as well as antitoxic immunity should be employed.

could be seen in the form of droplets and fatty acid crystals. The mean ether-extractable fat content (gravimetrically) of normal muscle samples was 7.3 per cent, that of gangrenous muscle samples 19.9 per cent, indicating a point of interest in view of the known effect of lecithinase on lipo-proteins.

When incubated with toxin, slices of rabbit or human muscle were disintegrated, while control slices incubated in toxin neutralized with antitoxin maintained their toughness for days. Toxin-treated muscle was a friable mass of loose and brittle fibers. The treatment of muscles with crystalline trypsin resulted in a semitransparent gelatinous, or white fibrous mass. Microscopically, these muscle-fibres could not be recognized, none the less they were tough and elastic. This gelatinous material, washed free of trypsin, could be completely lysed by toxin; conversely, the mass of fibre left after incubation with toxin could be almost completely digested by trypsin. These facts indicated the presence of two different substances in the muscle, the trypsin-soluble, toxin-insoluble protein of fibre, and the toxin-soluble, trypsin-insoluble frame-work on which the structural integrity of the muscle depends. These investigators reported that the toxin of *Cl. welchii* exercises a "collagenase" activity which was believed to be directly involved in the muscle destruction in gas-gangrene.

In connection with the characterization of certain proteolytic enzymes as collagenase, the nature of the substrate and the methods used for its preparation must be a consideration. Neuman and Tytell (1950) reported that collagens prepared by more drastic means and commercial hide powder were highly susceptible to attack by the proteolytic enzymes. Denaturation of collagen by heat and urea produces general susceptibility to common proteolytic enzymes. On the other hand, collagens from various sources prepared by mild processes designed not to alter the properties were found to be resistant to the action of trypsin, chymotrypsin, and papain. These collagens were readily attacked (solubilized) by the proteolytic enzymes of *Cl. histolyticum* and by pepsin. The proteolytic activity of *Cl. perfringens* filtrates are found to be 10 to 20 fold weaker than those of *Cl. histolyticum* filtrates in the degradation of collagen.

Collagen is a protein forming the chief constituent of connective tissue and the organic substance of bones. Collagenous fibres when boiled with water dissolve and yield a colloidal solution of animal glue

or gelatin. The collagenous fibres by X-ray diffraction methods (Astbury and Bell, 1940) are found to be composed of parallel bundles of long chains of polypeptides. The main features of the X-ray photographs of collagen fibres and oriented gelatin are reported to be the same (Astbury, 1943). The amino acid composition of collagen and gelatin is likewise reported to be essentially the same. Collagen has been reported to be digestible slowly in pepsin-hydrochloric acid solution but by trypsin only at temperatures above 40°C. or after previous action of pepsin in hydrochloric acid solution. The collagenous bundles, which are digestible by pepsin, are, however, resistant to alkaline trypsin solution. The collagenase activity of *Cl. welchii* toxin is found to be optimal at pH 5.5. The above facts may suggest that the "collagenase" activity of *Cl. welchii* may resemble that of pepsin. This activity is neutralized by *Cl. welchii* type A antisera.

Oakley, Warrack and van Heyningen (1946) reported collagenase as an additional toxin immunologically distinct from  $\alpha$ -toxin,  $\beta$ -toxin and hyaluronidase. This toxin which is capable of breaking down muscle fibres by attacking their collagen and reticulin scaffolding was believed to be responsible for the pulping of muscle seen in human gas-gangrene. They reported that *Cl. welchii* type A filtrates in which all the  $\alpha$ -toxin had been neutralized still disintegrated muscle, while filtrates in which all the collagenase ( $\kappa$ -toxin) was neutralized had no muscle disintegrating power, though a large amount of  $\alpha$ -toxin may still be present. In this connection it may be mentioned that Maschmann (1937) had previously reported that *Cl. welchii* toxin exercises proteolytic activity which was specifically neutralizable (or was inhibited) by antitoxic horse sera (p. 256).

In the evaluation of the above observations, the following findings are of interest. Evans (1947) stated that neither  $\beta$ -antihemolysin, anti-hyaluronidase, nor anti-collagenase enhance the protective properties of  $\alpha$ -antitoxin. Anti-serum containing  $\alpha$ -antitoxin and no anti-collagenase was found to be highly effective in protecting guinea pigs against infection, whereas an antiserum containing anti-collagenase but not  $\alpha$ -antitoxin was able neither to protect against infection nor enhance the protective properties of  $\alpha$ -antitoxin. While Evans recognizes that the rapid spread of the disease may be associated with hyaluronidase production and that muscle destruction may be a result of the action of collagenase, he does not find any evidence to support the view that

either of these enzymes plays any substantial part in the genesis of fatal gas-gangrene.

The findings of MacFarlane and MacLennan (1945) showed that gangrenous muscle samples contain 173 per cent more extractable fat than normal muscle. This may have a significant bearing on the fat catabolism in gas-gangrene. Frazer, *et al.* (1945) studied the effect of *Cl. welchii* toxin on tissue and fluid substrates *in vitro*, in animals, and in human subjects, with special reference to the effect of lecithinase on structural lipids. Direct local effects on connective tissue, striated muscle, adipose tissue, and peripheral nerve were described. The action of the toxin on red blood cells, chylomicrons, and plasma lipoprotein complexes was compared in guinea pig and man. Demyelination in the central nervous system was demonstrable *in vitro* and in experimental and clinical studies. Fat-embolism was produced experimentally by intramuscular or intraperitoneal injection of *Cl. welchii* toxin, and has been demonstrated in human postmortem material. The origin of the fat in the animal experiments was considered to be the site of local tissue destruction.

Pertaining to the reasons for the failure of preventive measures with the use of antitoxin the following observations are significant. Zamecnik and Lipmann (1947) found that the substrate lecithin interferes with the combining reaction of *Cl. welchii*  $\alpha$ -toxin (lecithinase) and  $\alpha$ -antitoxin. If the lecithinase and lecithin are brought together first, the antitoxin fails to inhibit the enzymatic reaction, but gradually decelerates it. If the lecithinase is brought together with a mixture of lecithin and antitoxin, it appears to combine in part with each, and the enzymic process takes place at a reduced rate, which gradually declines farther. If, on the other hand, the lecithinase is first brought into contact with antitoxin, before lecithin is added, the enzymatic reaction is completely inhibited. These facts show: (a) that both the substrate and the specific antitoxin compete for the same active site of the enzyme; (b) that the enzyme-substrate complex is dissociable; and (c) that toxin-antitoxin combination is relatively non-dissociable.





## Part V

# Antibodies Against Respiratory Enzymes

IN THE preceding pages the properties of antibodies against the proteolytic, lipolytic and amylolytic enzymes, etc. of bacteria were described. It is important from the standpoint of antibacterial immunity to consider whether there exist antibodies against the respiratory or oxidative enzymes of bacterial and other cells. A discussion of this question involves a brief survey of the chemical nature, distribution and properties of these enzymes, and of certain immunological facts about them. For fuller information standard books on enzymes should be consulted.

## A. RESPIRATORY ENZYMES

### 1. Dehydrogenases, Flavoproteins, Cocarboxylase Containing Enzymes, and Heme Containing Enzymes

*Dehydrogenases:* A dehydrogenase is a conjugated protein which consists of a specific protein and a prosthetic group common to all of the known dehydrogenases. At present there are known to exist *diphosphopyridine adenine dinucleotide* or coenzyme I, and *triphosphopyridine adenine dinucleotide* or coenzyme II. Since the coenzyme group is one or the other of these in all known cozymase dehydrogenases, certain specific characteristics of the proteins must regulate the activity of these enzymes. Several of these specific proteins have been obtained in crystalline form. We are entirely in the dark regarding the peculiarities of the specific protein molecule.

*Flavoproteins:* A flavoprotein is a conjugated protein which consists of a specific protein and a prosthetic group: at present there are known to exist *riboflavin phosphate*, a mononucleotide, and *riboflavin adenine dinucleotide* as the prosthetic groups of flavoproteins. Besides the rôle

they play in a complete respiratory system, flavoproteins also function as oxidases (dehydrogenases). This does not involve the participation of dehydrogenases containing coenzyme I or II. *d-Amino acid oxidase*, *l-amino acid oxidase*, *xanthine oxidase*, *aldehyde oxidase* and *fumaric hydrogenase*, a mold *glucose dehydrogenase*, *glycine oxidase* and *cytochrome reductase* have been shown to be flavoproteins containing either isoalloxazine-d-ribosephosphate, or isoalloxazine-adenine dinucleotide as the coenzymes.

**Fermentation:** Biological oxidation reactions which cause the reversible breakdown of glycogen to lactic acid in muscle in the absence of air, and similar reactions which are brought about by yeast, causing the breakdown of glucose to alcohol are known as fermentation reactions. Various enzymes and coenzymes which mediate the reactions involved in the fermentation reactions are given below.

**Enzymes Containing Cocarboxylase:** Cocarboxylase is an essential coenzyme of fermentation. In alcoholic fermentation (yeast, plant tissue and bacteria) pyruvic acid is decarboxylated by carboxylase, of which cocarboxylase (thiamine diphosphate) is the coenzyme group. In animal tissue on the other hand, pyruvic acid is reduced to lactic acid. This does not depend on the absence of cocarboxylase in animals but on the specificity of the enzyme, by which the oxidative decomposition is indicated. Cocarboxylase is likewise a component of the enzyme systems known as *pyruvic dehydrogenase*,  *$\alpha$ -keto-glutaric carboxylase*, *pyruvic ketolase* and *aldehyde ketolase*.

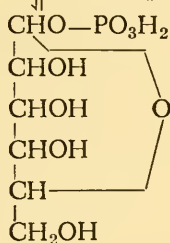
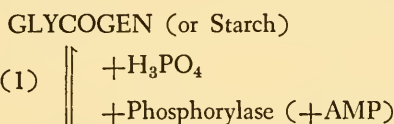
**Heme Containing Enzymes:** These catalysts participate in reactions which involve oxygen or hydrogen peroxide as one of the reactants. They all contain heme as the common prosthetic group in combination with specific proteins which regulate the specific activity of each catalyst. There are at least ten known heme containing catalysts. They are: hemoglobin, myoglobin, cytochrome *a*, *b*, *c*, cytochrome oxidase, cytochrome *c* peroxidase, catalase, peroxidase, and verdo-peroxidase. In most aerobic respirations the cytochrome oxidase system constitutes the terminal oxidizing system.

A complete aerobic respiratory system comprises the following parts: Dehydrogenase+substrate+flavoprotein+terminal oxidizing system.

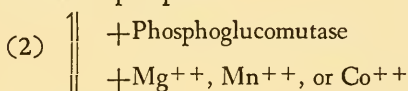
According to this scheme, the substrate is dehydrogenated (oxidized) by dehydrogenase whereby the coenzyme group (of dehydrogenase) is reduced. The reduced coenzyme is then dehydrogenated by a flavo-

protein yielding leucoflavoprotein. The latter reacts with an oxidizing system, oxygen directly, or through the mediation of the cytochrome-cytochrome oxidase system which constitutes a terminal oxidizing system.\*

## 2. Enzymes Involved in the Fermentation and Oxidation of Glucose (Phosphorylation of Hexoses and Oxidation-Reduction Reactions of Hexose-Breakdown Products)

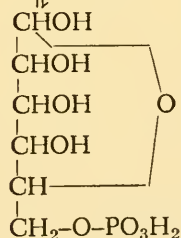


Glucose-1-phosphate



Glucose  
ATP  
 $\text{Mg}^{++}$   
Hexokinase

$\rightleftharpoons$  (3)



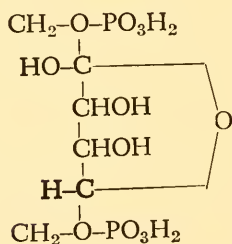
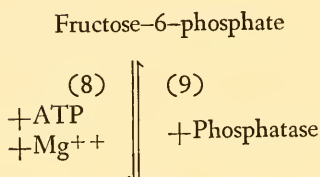
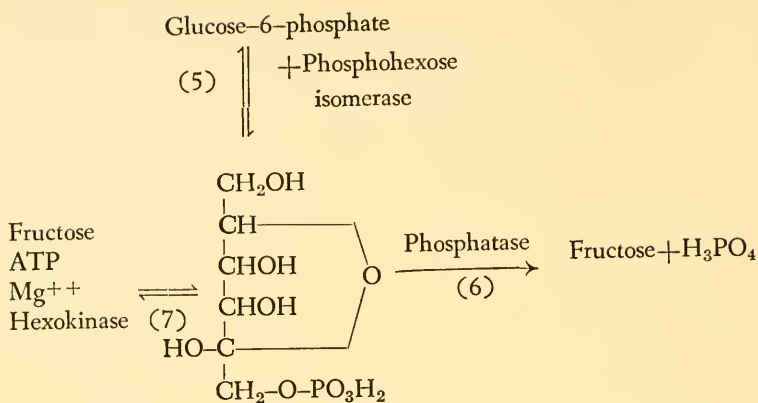
Glucose-6-phosphate

Phosphatase

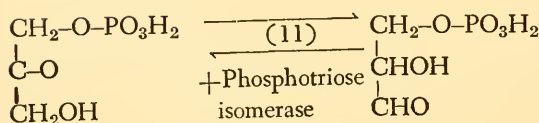
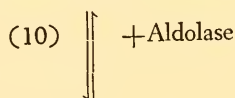
(4)

$\rightarrow$  Glucose +  $\text{H}_3\text{PO}_4$

\*For a comprehensive discussion of respiratory enzymes the reader is referred to: *A Symposium on Respiratory Enzymes* (1942), The University of Wisconsin Press; C. Oppenheimer and K. G. Stern (1939); and Sumner and Somers (1947).

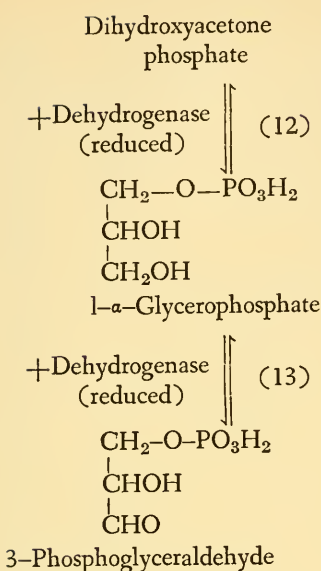


Fructose-1,6-diphosphate

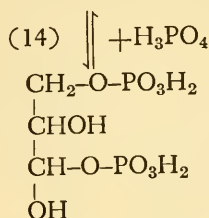


Dihydroxyacetone-phosphate

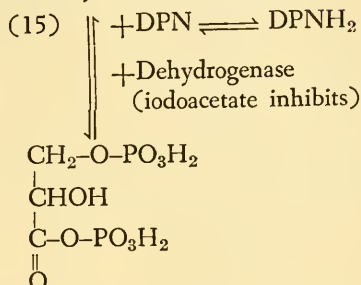
d-3-phospho-glyceraldehyde



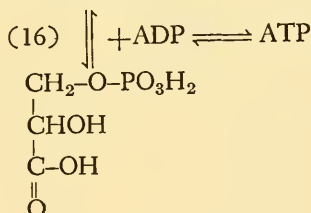
d-3-phosphoglyceraldehyde



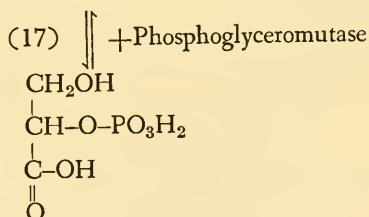
d-Glyceraldehyde-1,3-diphosphate



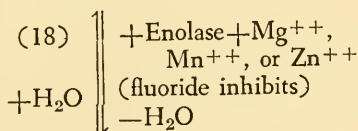
1, 3-Diphosphoglyceric acid



d-3-Phosphoglyceric acid

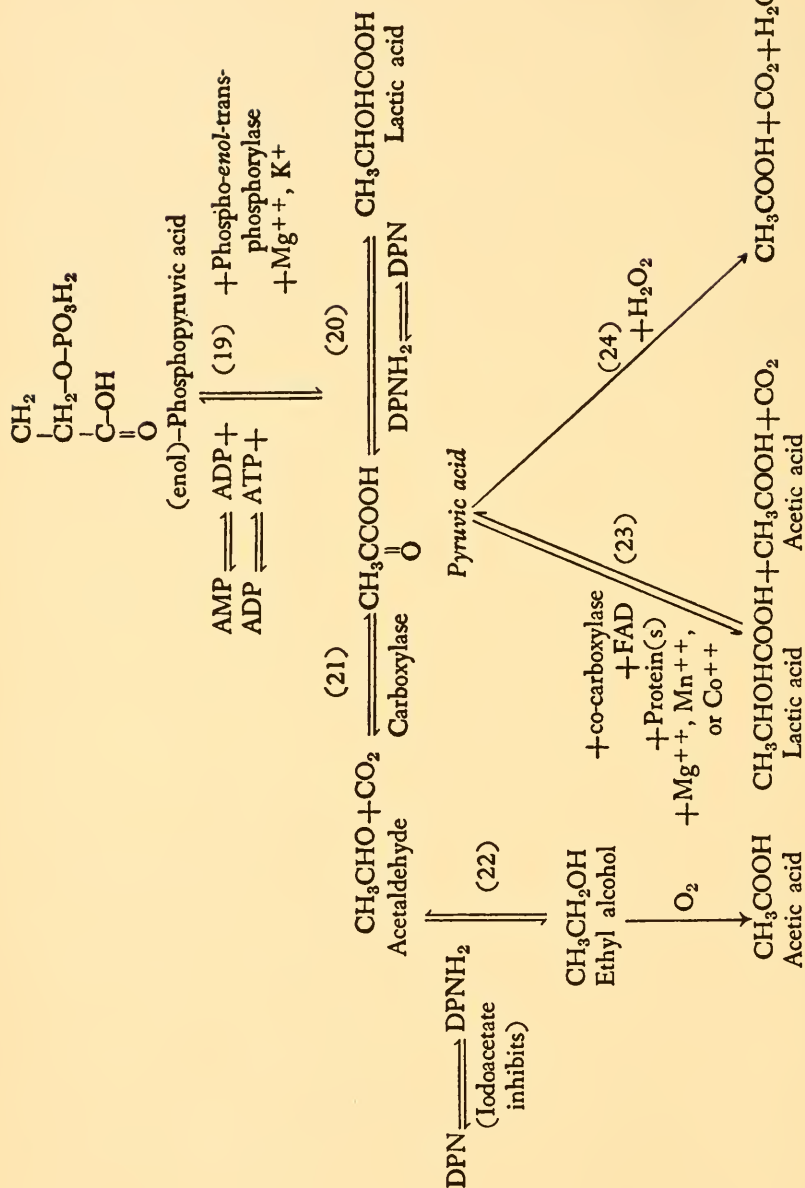


d-2-Phosphoglyceric acid



(enol)-Phosphopyruvic acid





AMP=Adenosine-monophosphate, or adenylic acid. ADP=Adenosine diphosphate. ATP=Adenosine triphosphate. DPN=Diphosphopyridine nucleotide. DPNH<sub>2</sub>=Reduced diphosphopyridine nucleotide. FAD=Flavine adenine dinucleotide.

All true ester phosphates involving phosphate esterification of alcoholic groups arise exclusively from transphosphorylation with the adenylic system or by intramolecular phosphate split. Inorganic phosphate is never taken directly into alcoholic groups, but only into carbonyl or carboxyl groups. On the other hand, energy-rich phosphate bonds are only created, directly or indirectly, by the oxidative reaction step in phosphorylated intermediates.

In the preliminary reactions, steps from (3) to (11), glucose is converted into a metabolizable form. There is neither oxidation nor reduction. In these reactions chemical work is done at the expense of the terminal energy-rich phosphate bonds of two molecules of ATP. The free energy changes, with the exception of the phosphorylation of the hexoses and fructose-6-phosphate, is comparatively small. The energy put into the glucose molecule is recovered in two subsequent steps of the reactions of hexose split products. (For an extensive discussion the reader is referred to Lipmann, 1941; Baldwin, 1947.)

In the oxidation-reduction step involving reactions (14), (15) and (16) nearly as much of the free energy of the oxidation of glyceraldehyde to the glyceric acid level is taken up by the phosphorylation of ADP to ATP ( $\Delta F=10,000$  to  $12,000$  calories/mole of phosphate) as is taken up for the reduction of DPN to  $DPNH_2$ . The former is a net gain of free energy, while the  $DPNH_2$  is reoxidized in the reduction of one mole of pyruvic to lactic acid. The free energy change in this reduction at pH 7 is about  $+8300$  calories.

The oxidation of phosphoglyceraldehyde to phosphoglyceric acid makes possible the formation of the second energy rich phosphate bond, which is produced by the dehydration of 2-phosphoglyceric acid to phosphopyruvic acid. In this change as much as  $8000$  calories per mole are liberated. Of this amount,  $2000$  calories are derived from an increase in entropy due to the formation of a  $C=O$  group.

The high potential energy of (*enol*)-phosphopyruvate resides in the fact that it is capable of converting ADP to ATP (reaction, 19). This change nets for ATP  $11,250$  calories/mole.

Thus, as far as the energy rich phosphate bond is concerned, two new molecules of ATP are gained for each molecule of glucose fermented. In the yeast fermentation of one mole of glucose,  $C_6H_{12}O_6 \rightarrow 2CO_2 + 2CH_3CH_2OH$ , the loss of free energy is about  $50,000$  calories. Two new energy-rich ATP molecules are formed at the cost of one

molecule of glucose. From the above relationships it is evident that about 40 per cent of the free energy liberated by one mole of glucose fermented is converted into energy utilizable by the living cell. The rest of the energy is dissipated in the form of heat.

*Description of the Reactions.*

- (1) In the presence of inorganic phosphate, glycogen or starch is reversibly converted into glucose-1-phosphate. This reversible reaction is catalyzed by *phosphorylase*. Adenylic acid functions as coenzyme. Reducing agents activate phosphorylase. Traces of glycogen are necessary as "priming" agent for the synthesis of glycogen from glucose-1-phosphate. Glucose inhibits phosphorylase activity.
- (2) *Phosphoglucomutase* converts glucose-1- to glucose-6-phosphate. The reaction is reversible;  $Mg^{++}$ ,  $Mn^{++}$ , or  $Co^{++}$  increase the activity of the enzyme.
- (3) *Hexokinase*, in the presence of ATP and  $Mg^{++}$ , phosphorylates glucose into glucose-6-phosphate. ATP serves as phosphate donator,  $ATP \rightleftharpoons ADP$ .
- (4) *Phosphatase* hydrolyzes glucose-6-phosphate, yielding glucose and inorganic phosphate.
- (5) *Phosphohexose isomerase* converts glucose-6-phosphate to fructose-6-phosphate.
- (6) *Phosphatase* hydrolyzes fructose-6-phosphate into fructose and inorganic phosphate.
- (7) *Hexokinase*, in the presence of ATP and  $Mg^{++}$ , phosphorylates fructose, yielding fructose-6-phosphate.
- (8) Fructose-6-phosphate is converted into fructose-1,6-diphosphate. ATP and  $Mg^{++}$  are required. The enzyme responsible for this reaction has not been adequately studied.
- (9) Fructose-1,6-diphosphate can be hydrolyzed into fructose-6-phosphate by *phosphatase*.
- (10) *Aldolase* reversibly splits fructose-1,6-diphosphate into dihydroxyacetone and d-3-phosphoglyceraldehyde.
- (11) *Phosphotriose isomerase* rapidly establishes the equilibrium between the two triose phosphates produced in reaction (10).
- (12) *Dehydrogenase* reversibly reduces dihydroxyacetone phosphate to l- $\alpha$ -glycerophosphate.

- (13) *Glycerophosphate dehydrogenase* reversibly oxidizes l- $\alpha$ -glycerophosphate to 3-phosphoglyceraldehyde.
- (14) d-3-Phosphoglyceraldehyde is reversibly phosphorylated, yielding d-glyceraldehyde-1,3-diphosphate, in the presence of inorganic phosphate (Meyerhof, 1941).
- (15) *Phosphoglyceraldehyde dehydrogenase* dehydrogenates 1,3-diphosphoglyceraldehyde to 1,3-diphosphoglyceric acid. Cozymase I (DPN) functions as hydrogen acceptor ( $\text{DPN} \rightleftharpoons \text{DPNH}_2$ ). This reaction is inhibited by iodoacetate.
- (16) 1,3-Diphosphoglyceric acid obtained in reaction (15) is enzymatically dephosphorylated to d-3-phosphoglyceric acid. ADP serves as phosphate acceptor ( $\text{ADP} \rightleftharpoons \text{ATP}$ ).
- (17) *Phosphoglyceromutase* converts d-3-phosphoglyceric acid to d-2-phosphoglyceric acid.
- (18) *Enolase* catalyzes the removal of one molecule of water from d-2-phosphoglyceric acid, yielding (*enol*)-phosphopyruvic acid. The reaction is reversible. The enzyme requires  $\text{Mg}^{++}$ ,  $\text{Mn}^{++}$ , or  $\text{Zn}^{++}$  for activation, probably, forming an active metal protein complex. Enolase is strongly inhibited by fluorides. The inhibition occurs in the presence of phosphate; a magnesium-fluorophosphate complex is formed which displaces the magnesium from the enzyme. A high concentration of pyrophosphate also inhibits enolase even in the absence of fluoride; here also, probably, a magnesium pyrophosphate complex is formed.
- (19) *Phospho-enol-transphosphorylase* reversibly dephosphorylates (*enol*)-phospho-pyruvic acid to pyruvic acid.  $\text{Mg}^{++}$  is required,  $\text{K}^+$  is essential (Lardy and Ziegler, 1945), AMP or ADP serves as phosphate acceptor ( $\text{AMP} \rightleftharpoons \text{ADP}$ ;  $\text{ADP} \rightleftharpoons \text{ATP}$ ). Calcium ion inhibits the reaction.
- (20) In the muscle, pyruvic acid is reversibly reduced to lactic acid. Cozymase I-dehydrogenase catalyzes this reaction.  $\text{DPNH}_2$  produced in reaction (15) can readily serve as hydrogen donator, producing lactic acid.
- (21) In yeast, pyruvate is decarboxylated by carboxylase (Thiamine-diphosphate as coenzyme) to acetaldehyde.
- (22) Acetaldehyde formed in reaction (21) is reversibly reduced to ethyl alcohol by means of alcohol dehydrogenase

(Cozymase-I  $\rightleftharpoons$  reduced Cozymase). The enzyme is inhibited by iodoacetate.

- (23) In bacteria, pyruvic acid can undergo dismutation yielding lactic and acetic acids (Lipmann, 1939; Krebs, 1937). According to Lipmann, the reaction requires cocarboxylase, flavine-adeninedinucleotide,  $Mg^{++}$ ,  $Mn^{++}$ , or  $Co^{++}$ , inorganic phosphate and protein(s).
- (24) The decarboxylation of pyruvate by means of hydrogen peroxide to acetic acid, water and carbon dioxide occurs in pneumococci (Sevag, 1933) and certain other bacteria. Hydrogen peroxide is formed from the aerobic oxidation of glucose, lactate, etc. in these bacteria. (For further information see Sumner and Somers, 1947.)

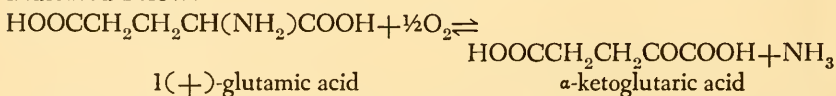
### 3. Tricarboxylic Acid Cycle\*

From the standpoint of the synthesis of amino acids, and therefore of proteins, pyruvic acid would seem to be the most critical  $\alpha$ -keto acid as the starting point. The following reactions (p. 373) are given to show how higher  $\alpha$ -keto acids can be derived from pyruvic acid.

(For the factors involved in the above reactions, see Werkman and Wood, 1942; Lipmann, 1941; Barron, 1943; Krebs, 1943; Sumner and Somers, 1947; Baldwin, 1947; Gurin, 1948).

### 4. Synthesis of Amino Acids From $\alpha$ -Keto Acids

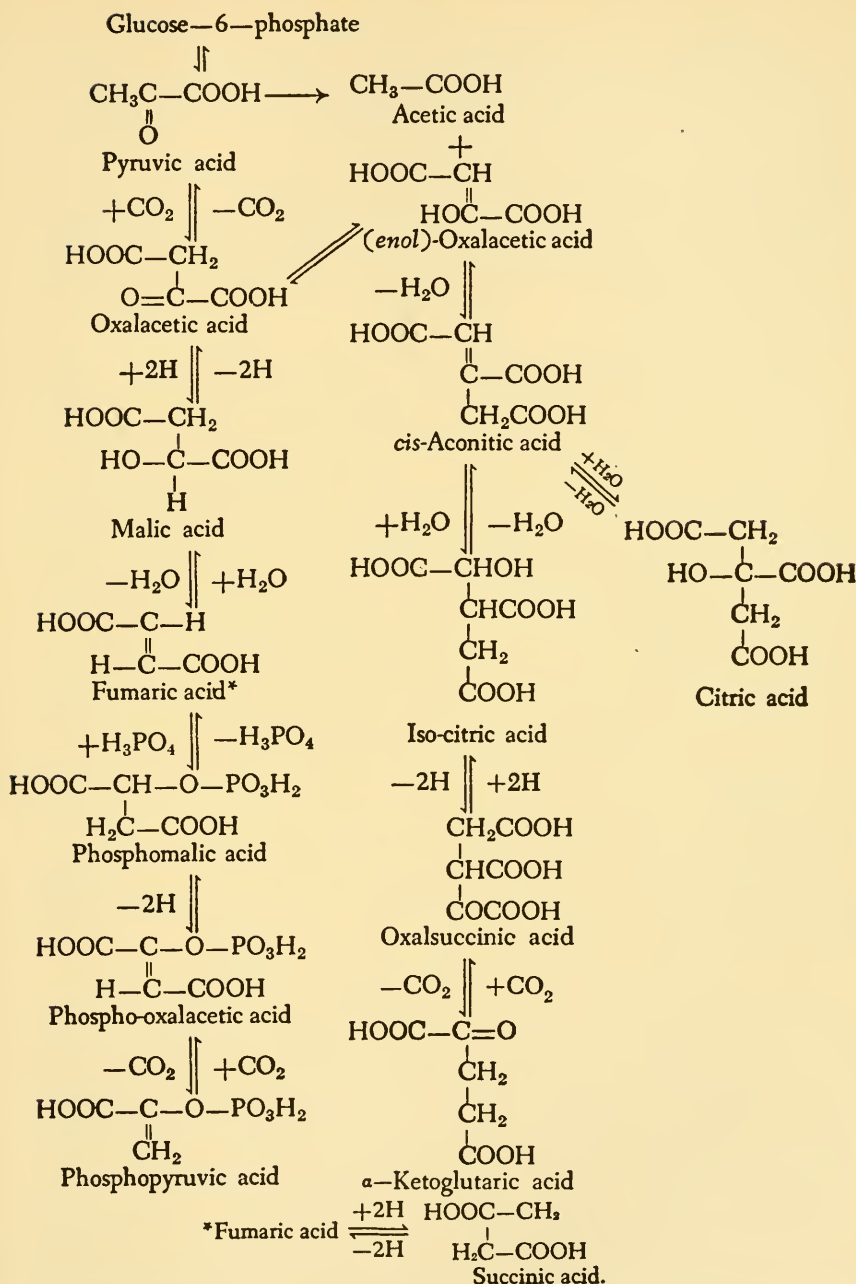
**a. Oxidative Deamination of Amino Acids.** Two principal types of reactions are responsible for the synthesis of amino acids. The synthesis of amino acids can take place by reversible amination of  $\alpha$ -keto acids derived from the intermediary products of carbohydrate metabolism, and from the oxidative deamination of amino acids as indicated below:



The enzyme which catalyzes this reaction is known as glutamic dehydrogenase and is found in yeast, plants, bacteria and animals. Euler, *et al.* (1938) and Dewan (1938) found this enzyme in liver,

\*Or Krebs' citric acid cycle has been undergoing, with respect to the sequence of



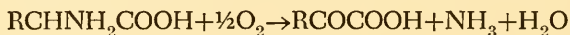


intermediates, etc., constant metamorphoses; the most recent view favors Krebs' original scheme. (For the latest review see S. Ochoa, *Phys. Revs.*, 31:56-106, 1951.)

kidney and heart. It requires cozymase I for oxidative deamination, and dihydro-cozymase, or a hydrogen-donating system producing dihydro-cozymase, for reductive amination of  $\alpha$ -ketoglutaric acid. Methylene blue, pyocyanine, flavin-phosphate, flavoprotein (yeast), and cytochrome *a* and *b* preparations are found to function as carriers to varying degrees. According to Euler, *et al.* (1938) glutamic acid most readily transfers its amino group to  $\alpha$ -keto acids forming new amino acids. Krebs and Cohen (1939) described the formation of glutamic acid together with succinic acid oxidatively from ammonium salts and  $\alpha$ -ketoglutaric acid.

Green, *et al.* (1943) and Blanchard, *et al.* (1944) obtained highly active enzyme preparations from the kidney of rabbit, cat, mouse and pig which catalyzed the oxidation of the following twelve amino acids to the corresponding  $\alpha$ -keto acids. Given in the descending order of oxidation velocities, these amino acids are: leucine, methionine, norleucine, norvaline, phenylalanine, tryptophane, isoleucine, tyrosine, cystine and valine, histidine (oxidative splitting of the ring) and alanine. It had little, if any, action on aspartic acid, glutamic acid, arginine, ornithine, lysine, serine and threonine, and no action on  $\beta$ -alanine, glycine or d-amino acids. Green, *et al.* (1944) and Blanchard, *et al.* (1945) showed that l-amino acid oxidase isolated from rat kidney is a flavoprotein which oxidizes both dihydrocozymase I and l-amino acids. This flavoprotein contains flavinmonophosphate as prosthetic group which is not identical with flavin adenine dinucleotide.

Bernheim, *et al.* (1935) and Stumpf and Green (1944) reported that suspensions of young cultures of *Proteus vulgaris* oxidize practically all the known natural amino acids in the following manner:



Suspensions of cells which were kept at 0° for two weeks lost part of their activity and were capable of oxidizing only eleven of the 22 amino acids which were attacked originally. It was, therefore, concluded that there are at least several enzymes which are involved in the oxidation of 22 amino acids by young suspensions of this organism.

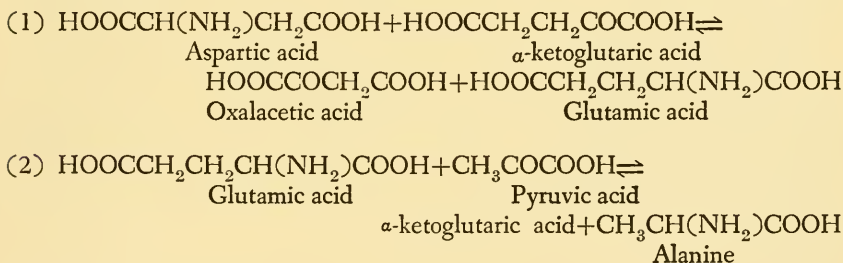
Cell-free extracts prepared from *Proteus vulgaris* (Stumpf and Green, 1944), like the aged suspensions of whole cells, were found to be incapable of oxidizing dl-serine, l-aspartic acid, l-glutamic acid, dl-alanine, dl-valine, l-proline, dl-threonine, l-ornithine, l-lysine, and

glycine. Fresh cell-suspensions were found not only capable of oxidatively deaminating alanine, serine, threonine, glutamic acid and aspartic acid to their corresponding  $\alpha$ -keto acids, but the latter were further oxidized.

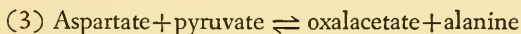
The equivalent of the *Proteus* enzyme was found in washed cell suspensions of *Aerobacter aerogenes* and *Pseudomonas pyocyaneus*, but not in those of *E. coli*, *Streptococcus hemolyticus*, *Diplococcus pneumoniae*, *Salmonella paratyphi*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Sarcina lutea*.

It is apparent that  $\alpha$ -keto acids derived in a manner described above can serve as bases for the synthesis of different  $\alpha$ -keto acids which can be aminated forming new amino acids.

**b. Synthesis of Amino Acids by Transamination.** Amino acids can also be synthesized by the transfer of an amino group from an amino acid to an  $\alpha$ -keto acid in the following manner:



Two separate enzyme systems mediate the above reactions. When these two enzyme systems are present as a mixture, the reaction,



also takes place (Green, Leloir and Nocito, 1945; O'Kane and Gun-salus, 1947). These investigators did not find an enzyme system specific for reaction (3).

It is, therefore, claimed that there are two principal transaminases: (1) the aspartic-glutamic enzyme system, and (2) the alanine-glutamic enzyme system, which function reversibly as indicated above. (For extensive discussion of transamination reactions see Cohen, 1942, and Braunstein, 1947.)

In the animal system, the most active transamination enzyme system is claimed to comprise the three paired reactions described above. Of

these three, the most active pair is found to be the glutamic-aspartic transaminase system (Cohen, 1942; Braunstein, 1947).

In bacteria, the following principal transamination reactions are reported (Lichstein and Cohen, 1945):

- (1) 1(+)-glutamic acid + oxalacetic acid  $\rightarrow$   
 $\alpha$ -ketoglutaric acid + 1(-)-aspartic acid
- (2) 1(-)-aspartic acid +  $\alpha$ -ketoglutaric acid  $\rightarrow$   
 $\alpha$ -ketoglutaric acid + 1(+)-glutamic acid + oxalacetic acid
- (3)  $\alpha$ -ketoglutaric acid + 1(+)-alanine  $\rightarrow$  1(+)-glutamic acid + pyruvic acid

Both reactions (2) and (3) are said to proceed very slowly as compared with reaction (1) which shows activity of a high order of magnitude. They found that *E. coli*, *B. dysenteriae* (Shiga), *B. typhosus*, *B. proteus*, *B. pyocyaneus*, *Azotobacter vinelandii*, *Staphylococcus aureus* and *albus*, *Cl. welchii*, *Streptococcus hemolyticus* and *viridans*, and *Pneumococcus*, Type I, were active in catalyzing these reactions.

The data at present are inadequate concerning the question of whether or not transamination reactions involve reversible oxidative deamination. Following the work of Snell (1945), and Schlenk and Snell (1945) indicating a role for pyridoxal and pyridoxamine in transamination, Lichstein, Gunsalus and Umbreit (1945, 1947) demonstrated that pyridoxal phosphate functions as the coenzyme of glutamic-aspartic transaminase of bacterial cells.

Snell (1945) postulated that the prosthetic group, pyridoxal, of transaminase acted by alternately accepting and donating amino groups, but it has not as yet been established conclusively that this cyclical amination and deamination of the prosthetic group, pyridoxal phosphate, occurs during the process of transamination. Umbreit, O'Kane, and Gunsalus (1948) presented data in favor of and against this concept. In their study, the activities of pyridoxal phosphate and "pyridoxamine phosphate" on transamination reactions were compared.

In the generation of pyruvate from carbohydrate metabolism, and the initiation of the tricarboxylic acid cycle via pyruvate, three most important  $\alpha$ -keto acids are formed. From these, three reactive amino acids, alanine, aspartic acid and glutamic acid, are formed. These amino acids in conjunction with  $\alpha$ -keto acids derived by various means can serve as a basis for the synthesis of other amino acids by any one of the processes outlined above, paving the way for the synthesis of proteins and other

critical cellular components required for growth and multiplication. (For a comprehensive integration of the genetic relationships of amino acids and the metabolic functions of the dicarboxylic acid system, the reader may be referred to Braunstein, 1947.)

## B. PROBLEMS DEALING WITH THE PRODUCTION OF ANTIBODY AGAINST THE RESPIRATORY ENZYMES

In the preceding pages it has been shown that the *dehydrogenases* so far described contain identical prosthetic groups (coenzyme I or II). Similarly the prosthetic group (riboflavin mononucleotide or riboflavin adenine dinucleotide) of the *flavoproteins*, the prosthetic group (adenylic acid) of *phosphorylases*, the prosthetic group (cocarboxylase, thiamin diphosphate) of *carboxylases*, the prosthetic group (iron porphyrin, heme) of the ten different heme containing enzymes are respectively identical. No matter what the source of these enzymes, the respective prosthetic groups are chemically the same.

The general question as to whether or not it is possible to produce antibody against the respiratory enzymes evolves into two parts:

(a) Is it possible to produce antibodies to respiratory enzymes which contain prosthetic groups which are natural to the host? If antibodies are produced, will these react against the prosthetic groups as well as the protein moiety?

(b) Is it possible to immobilize the activity of the prosthetic groups by combination between the protein component of the enzyme and specific antibody?

A satisfactory answer to this important biological question requires further critical experimentation. The question as to whether or not the carboxylase-anti-carboxylase combination inactivates carboxylase must be answered.\* Does the oxygen combining capacity of hemoglobin in combination with its homologous antibody undergo diminution? Similarly, does the catalase-anticatalase combination produce the inactivation of catalase, etc.? These are questions which must be answered experimentally. The answers will no doubt have direct bearing on understanding of the mechanism of immune protection.

\*In collaboration with the author, Drs. V. Z. Pasternak and Ruth E. Miller found that immune sera prepared against isolated yeast carboxylase, or dry whole yeast, or live whole yeast markedly inhibited the activity of the isolated yeast carboxylase (J. Bact., 61, No. 2, 1951).



As discussed in Part I of this treatise, precipitating and anaphylactic antibodies have been produced against crystalline catalases. As in the above serological studies with hemoglobin, the heme, the prosthetic group of catalase, did not react with anti-beef catalase either in precipitation or in inhibition tests. Beef hemoglobin as test antigen also failed to give any reaction, showing that the antibody is specific only to catalase, and anti-heme is absent in the anti-catalase serum.

A few investigators have sought to determine whether the activity of catalase is lowered when combined with catalase antibody. Campbell and Fourt (1939) reported hardly any loss of activity if the mixture of antigen and antiserum was diluted directly, and a variable loss if the precipitate was resuspended by ordinary shaking and stirring, after having been centrifuged. The evidence appears to be inconclusive in view of the possible dissociation in dilute reaction mixtures. Harkins, *et al.* (1940) using multilayer technique concluded that the undissociated catalase-anticatalase compound shows enzymic activity. The activity per gm. of adsorbed catalase was only a fifth to a tenth of that in solution. This observation was considered by the authors as tentative for two reasons: (1) the Kat. f. (activity) in solution is lower than for some solutions of crystalline catalase, which indicates the presence of denatured protein or an impurity which might be preferentially adsorbed, reducing the activity on the plates; (2) the effect of drying and aging may have impaired the activity, independent of the effects of adsorption alone. In view of the possible interference of the factors considered by these investigators the question as stated above remains unanswered.

It is generally known that those conjugated foreign proteins which contain prosthetic groups common to the species of animal undergoing immunization do not stimulate the formation of specific antibody against the prosthetic group. Antibody is produced against the protein moiety. Heidelberger and Landsteiner (1923) produced specific antibody against crystalline horse oxy-hemoglobin. In precipitation tests the immune serum reacted species specifically with homologous antigen, and somewhat weakly with the hemoglobin of the closely related donkey; it failed to react with the hemoglobin solutions of pig, ox, sheep, goat, rabbit, guinea pig, rat, mouse, chicken

and pigeon. A solution of hematin, the prosthetic group common to all the hemoglobins tested, caused no inhibition of the reaction between horse hemoglobin and the homologous immune serum. Hematin also failed to react with immune sera. These facts show that the heme group in hemoglobin does not function as a hapten. For this reason, the hemoglobin solutions of the various species of animals which contain the same heme group failed to cross-react with the anti-horse oxyhemoglobin serum.

Hektoen, *et al.* (1928) prepared solutions of dog muscle myoglobin ("muscle hemoglobin") free from blood hemoglobin. Serologically, myoglobin was sharply differentiated from the hemoglobin of dog blood.

The above cited studies show clearly that immune sera against hemoglobins, catalases or myoglobin do not contain anti-heme antibody. It is therefore obvious that heme, being a common prosthetic group of all the hemoglobins and hemin type of cellular and humoral enzymes, is incapable of stimulating the formation of antibody.

A carbohydrate isolated from  *$\beta$ -hemolytic streptococcus* was found to be serologically inactive (Kendall, *et al.*, 1937). This carbohydrate was found to be a common constituent of synovial fluid, vitreous humor, etc. for which reasons antibody against this carbohydrate was not found in anti-streptococcal sera.

It may be mentioned that sugars, such as glucose, galactose and lactose, etc. combined with p-aminophenol through an ether linkage and coupled with proteins through an azo group, stimulate the formation of antibodies. These sugars *per se* are common to the animal system; however, since they constitute a component of an artificial antigenic complex, they function as antigenic haptens. Aminobenzoyl derivatives of certain peptides such as glycyl-glycine, glycyl-leucine, leucyl-glycine, etc., coupled with proteins through an azo linkage have also been shown by Landsteiner and his associates to stimulate the formation of specific antibodies. These may also be explained in the above manner by assuming that they behave in the animal system apparently as foreign substances.

The principles underlying the above cited facts may no doubt apply to dehydrogenases, flavoproteins, carboxylases, phosphorylases, etc.,

since the prosthetic groups (as well as the corresponding vitamin derivatives) are common constituents of living cells. Failure to bear these facts in mind leads to erroneous conclusions. In this respect the following example is of interest.

## 1. Reasons for the Failure to Produce Antibody Against Yellow Enzyme

Varterész and Kesztsyüs (1940) studied the antigenic properties of yellow enzyme prepared according to the method of Warburg and Christian (1933). A 2.7 per cent solution of the enzyme was used to immunize 10 rabbits. Each rabbit was intravenously injected successively with 50, 100, 150, and 200 mg. of yellow enzyme at five day intervals. Twenty to twenty-five days later the rabbits were bled and the sera tested. The effect of the immune sera on the oxygen uptake of the following respiratory system was studied:

Specific protein from yeast (Zwischen Ferment)+hemolyzed horse red blood cells (apparently as source of coenzyme II)+hexose monophosphate+yellow enzyme+immune rabbit serum.

In the absence or presence of immune serum the same volume of oxygen was consumed. Substitution of oxygen-uptake measurement by the measurement of the time of decolorization of methylene blue likewise did not show any effect by immune serum.

These investigators did not report having performed precipitation, complement fixation or anaphylactic tests to demonstrate whether or not they succeeded in producing antibody against the enzyme at all. They explained the failure of any inhibitory effect on the oxygen consumption or methylene blue decolorization by immune serum by stating that "the protein of the yellow enzyme is not foreign to any species of animals." "The structure of the protein of the yellow enzyme is identical in all the species." These statements are in direct contradiction of the known facts that the specificities of flavo-proteins, etc. are related to the specific characteristics of the protein components. The failure of the above investigators to demonstrate any inhibitory effect by the sera of rabbits injected with their "yellow enzyme" would appear to be due to an unsatisfactory immunization and serological technique.

## 2. Inhibition of Pyruvic Acid Reductase by Specific Immune Serum

In Warburg's Laboratory, Kubowitz and Ott (1943) isolated in crystalline form from rat Jensen-sarcoma and rat muscle the specific protein of pyruvic acid reductase. From 3000 g. of tumors (ca. 600 g. dry weight) which were harvested from about 3000 rats about 50 mg., and from 1000 g. of muscle (ca. 200 g. dry weight), which were harvested from 10 to 12 rats, about 200 mg. of pure enzyme protein were obtained. The crystalline proteins were indistinguishable with respect to crystalline form and catalytic activity; furthermore, antibody against the tumor enzyme protein inhibited the catalytic activity of the enzyme protein obtained from rat muscle, and *vice versa*.

These enzyme proteins catalyzed the following reaction:

Pyruvic acid + dihydro-coenzyme I  $\rightleftharpoons$  lactic acid + coenzyme I. Under the conditions of the experiments the reaction in the above equation, measured spectroscopically, proceeded completely from left to right. As a result of the completeness of the reaction from left to right, the absorption band at  $\lambda 334 \text{ m}\mu$  shown by dihydro-coenzyme I disappeared completely. The diminution of the absorption of light thereby served as a measure of the enzyme activity of the test solutions.

Intravenous injections of rabbits with 6 mg. of enzyme protein at intervals of three days during two to three months produced anti-enzyme immunity. The blood of immunized rabbits was introduced into citrate solution (0.7 per cent sodium chloride containing 1.1 per cent sodium citrate) in which the final ratio of blood to citrate solution was three to one.

To determine the anti-enzyme activity of the immune plasma the following combinations were studied: enzyme protein without plasma; enzyme protein + normal plasma; enzyme protein + immune plasma, and immune plasma, without enzyme protein. The test solution consisted of a mixture of 2 $\gamma$  of enzyme protein (dissolved in 0.05 ml. of 0.02 M phosphate of pH 7.4) + 1.2 ml. of plasma, incubated at 20° for 10 minutes. To this was added 5.2 ml. of 0.1 M phosphate of pH 7.3 + 6.4 ml. of distilled water + 0.1 ml. of 0.01 M sodium pyruvate (final concentration =  $1 \times 10^{-4}$  M). At 0 hour 0.1 ml. (0.265 mg.) of dihydro-coenzyme I (final concentration =  $3.08 \times 10^{-5}$  M) was added



to the reaction mixture. Final total volume=13.0 ml.; pH=7.45; the depth of the reaction solution=2.93 cm.

The results of measurements were as follows: (a) normal rabbit plasma did not inhibit the activity of the enzyme, (b) immune plasma exercised inhibition, and (c) immune plasma against rat tumor enzyme protein and that against rat muscle enzyme protein exercised identical inhibitions against one or the other enzyme proteins.

The degrees of inhibition were dependent on the concentration of pyruvate. In the presence of  $1 \times 10^{-4}$  M pyruvate the inhibition was 75 per cent. Increasing the concentration to  $20 \times 10^{-4}$  M the inhibition was reduced to 45 per cent. This observation has experimental as well as theoretical significance. It would appear that the anti-reductase competes with the substrate pyruvate (possibly also with the dihydrocoenzyme I) for the same active group of the enzyme protein (or the combining site of the antigen). The results would indicate that antibody displaces pyruvate (possibly also the coenzyme) from its combination with the enzyme. However, another possible explanation of the above inhibition would be that the combination of the enzyme with the antibody occurs at sites other than that involved in the enzyme-substrate reaction. This combination may offer steric hindrance to the free access of substrate to the site of enzyme activity, or merely reduce the affinity of the enzyme complex for its specific substrate.

### 3. Inhibition of Yeast Hexokinase by Homologous Antiserum

As a continuation of the studies reported by Sevag and Miller (1948), Miller, Pasternak and Sevag (1949) investigated the effect of homologous rabbit immune serum on the activity of hexokinase isolated from yeast.

It is known (von Euler and Adler, 1935; Meyerhof, 1935; Colowick and Kalckar, 1943) that hexokinase catalyzes the transfer of one phosphate group from adenosine-triphosphate to glucose with the formation of glucose-6-phosphate. In this reaction an alcohol hydroxylic hydrogen is converted to a more acidic hydrogen.

The effect of anti-hexokinase rabbit serum on the formation of the acidic hydrogen in this system was determined. As will be seen below,



immune serum added to the complete reaction system at the beginning of the experiment or after the reaction has started, completely inhibits the activity of hexokinase.

Hexokinase was prepared from Fleischmann's baker's yeast according to the method of Kunitz and McDonald (1946) and the dialyzed "0.72" fraction was used for immunization and for activity tests with various sera (for details, see original literature). The method used for the measurement of hexokinase activity consisted of manometric measurement of carbon dioxide evolved according to the method Colowick and Kalckar (1943) and Berger, *et al.* (1946) adapted to our conditions.

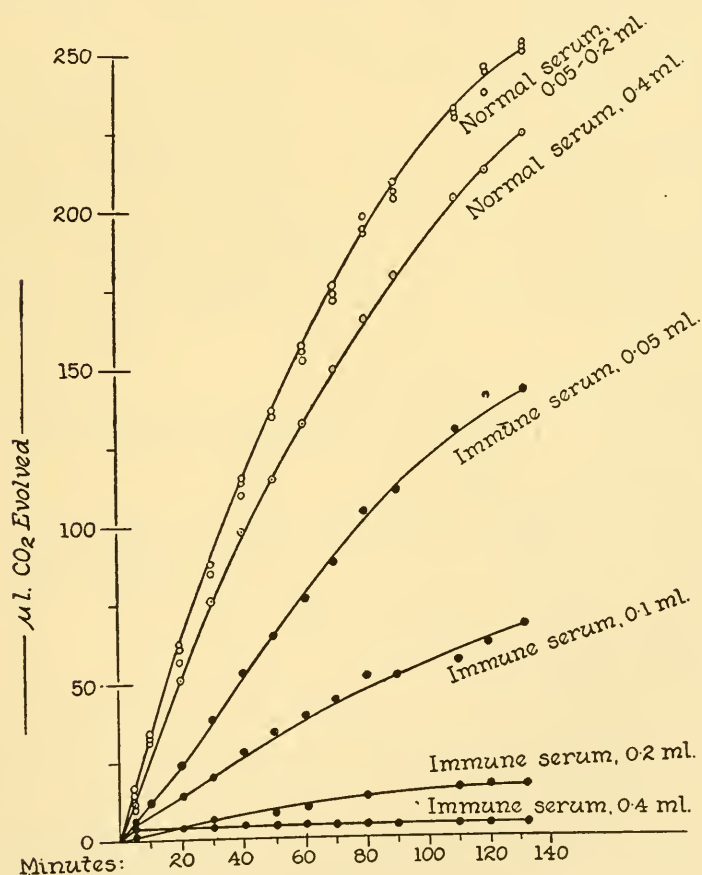


Fig. 1. Inhibitory effect on yeast hexokinase by homologous rabbit antiserum added before the reaction had started.

The results presented here show that rabbit immune serum prepared against partially purified yeast hexokinase completely inhibits its activity.

The high specificity of this reaction is indicated by the fact that: (a) a rabbit immune serum prepared against the same enzyme preparation suspended in Falba-mineral oil, even though it produced an immune serum with higher precipitating and complement-fixing titer, was incapable of exercising an inhibitory effect on the hexokinase activity; (b) a rabbit immune serum prepared against streptococcal hyaluronidase likewise failed to inhibit hexokinase activity; and (c) an aqueous extract of rat brain (8 ml. of chilled distilled water per rat brain) as

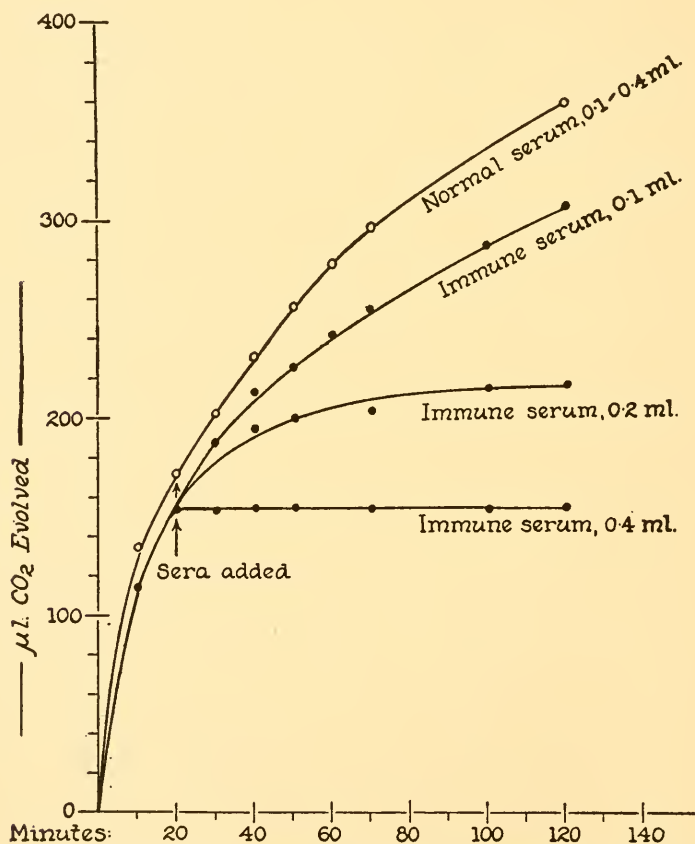


Fig. 2. Inhibitory effect on yeast hexokinase by homologous rabbit antiserum added after the reaction had proceeded for 20 minutes.

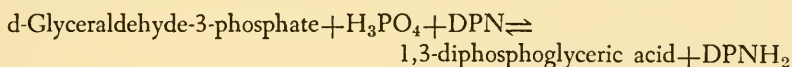
source of mammalian hexokinase when substituted for yeast hexokinase was not inhibited by rabbit normal and antiyeast hexokinase sera. Three-tenths ml. of brain extract was approximately equivalent in activity to 0.1 ml. of our yeast hexokinase preparation.

Four-tenths ml. of immune serum completely inhibits the activity of 0.1 ml. of hexokinase (0.1 mg. protein) solution. The inhibitions by 0.05 ml., 0.1 ml. and 0.2 ml. of immune serum are respectively, 42%, 73% and 94% (Fig. 1). The data presented in Fig. 2 show similar qualitative relationship with respect to the inhibition with various volumes of immune serum. The optimal volume (0.4 ml.) of immune serum added at a time when hexokinase activity was at its peak brought about an immediate inhibitory effect which persisted through the two hour period. This relationship between an enzyme and its homologous antibody would appear to be analogous to the inhibition of enzymes with specific inhibitors.

According to our unpublished data immune serum prepared against the whole yeast cell was found to produce 79% inhibition of the activity of dialyzed hexokinase preparation. Due to technical difficulties, the effect of the immune serum prepared against the isolated hexokinase on the hexokinase activity of the whole yeast cell has not as yet been determined.

#### 4. Inhibition of Yeast d-Glyceraldehyde-3-phosphate Dehydrogenase by Specific Anti-Serum

Krebs and Najjar (1948) produced specific antibody in rabbits to the yeast dehydrogenase involved in the reversible oxidation of d-glyceraldehyde-3-phosphate:



For immunization of the rabbits, the electrophoretically nearly homogeneous crystalline enzyme was used, but some of the animals received injections of the enzyme solution immediately before crystallization. The enzyme preparation contained DPN apparently bound to protein (cf. Taylor, Velick, Cori, Cori and Slein, 1948). Cysteine was necessary to obtain maximum activity.

Four medium sized rabbits were given a series of five subcutaneous

injections of 8 mgm. of the yeast dehydrogenase at 3-day intervals. The crystalline enzyme, stored as a suspension of crystals in alkaline ammonium sulfate solution, was dissolved in water and injected immediately. Sera were obtained two weeks after the last injections. Stronger antisera were obtained after a second series of injections two months later. Two rabbits received as many as four series of injections. Chickens were similarly used for the production of specific anti-enzyme serum.

Antibody levels of the sera were measured by the precipitin reaction, using the conventional ring test, and by the inhibition of activity of a given amount of the yeast dehydrogenase. Enzyme activity was followed by following the reduction of diphosphopyridine nucleotide (DPN) spectrophotometrically at 340 m $\mu$ . The per cent inhibition of 15  $\mu$ g. of crystalline enzyme was determined using 0.05 ml. of anti-serum. There was a fair correlation between the precipitin titer and the degree of inhibition by antisera.

Rabbit antisera to the yeast dehydrogenase did not inhibit the activity of hexokinase isolated from the same strain of baker's yeast. Rabbit and chicken antisera to the yeast dehydrogenase did not exercise any inhibitory effect on dehydrogenase of muscle.

One-tenth ml. of enzyme solution containing 15  $\mu$ g. of protein was added to a reaction mixture (in the Beckman cell) consisting of 2.5 ml. of cysteine-pyrophosphate buffer and 0.2 ml. of DPN (final concentration= $1.27 \times 10^{-7}$  moles per ml.) at 25°. The reaction was started by the addition of 0.2 ml. of a fresh 1:1 mixture of 5.3 per cent sodium arsenate and triose phosphate (final concentration= $1.27 \times 10^{-7}$  moles/ml.). Unless otherwise stated, antiserum was added to the above reaction mixture. Bimolecular rate constants were calculated

from the equation: 
$$K = \frac{1}{t} \frac{x}{a(a-x)}$$

a=initial concentration of triose phosphate and DPN

x=the amount of reduced DPN formed in time  $t$  (minutes)

K was found to decrease with time, but K values based on one minute readings were proportional to enzyme concentration. The per cent inhibition of enzyme activity was calculated from the one minute mixture.

Antigen-antibody combination, measured by the extent of inhibition,

was complete within the 10 minute period allowed. The turbidity developing in the Beckman cell due to antibody-enzyme flocculation also reached a maximum during this period. The absorption at 340  $m\mu$  due to the presence of a precipitate was small and was corrected for the initial reading.

It was found that with increasing amounts of antiserum the degree of inhibition increased almost linearly up to about 80 per cent. Beyond this the inhibition leveled off, never reaching complete inhibition. The residual enzyme activity in the presence of an excess amount of antibody suggested that the insoluble enzyme-antibody complex itself retained activity amounting to about 10 per cent of the original enzyme activity. The removal of the precipitate by centrifugation left a supernatant fluid with no enzyme activity, while the washed precipitate showed an activity only slightly lower than the residual activity.

*Effect of Diphosphopyridine nucleotide (DPN) on Enzyme-Antibody Combination.* As discussed above the presence of an excess amount of antiserum in the reaction system caused up to 95 per cent inhibition of the enzyme activity. The residual 5–10 per cent activity was that of the enzyme-antibody precipitate, which showed no evidence of dissociation at increasing concentration of DPN and triosephosphate.

In systems where only 0.05 ml. of antiserum was added to the reaction mixture containing DPN, enzyme, buffer etc., the activity determined after 5 minutes incubation showed 31 per cent inhibition of the enzyme. Longer incubation did not increase the extent of inhibition. When DPN was absent during the preliminary incubation of antiserum and enzyme, there was found 54 per cent inhibition of activity. The substrate triosephosphate had no influence on this relationship among the three reactants, coenzyme DPN, enzyme and antibody.

The rate of combination of enzyme with antibody was determined while the enzyme was acting on its substrate. High concentrations of triosephosphate and DPN were used, and under these conditions the rate was found to be nearly linear after the first half minute. Rabbit antiserum in deficient and excess amounts was added to identical reaction mixtures at the third minute and the rate followed until it again became linear, indicating completion of enzyme-antibody combination. Under these conditions, the difference between the control rate and the residual rate of the system containing an adequate amount



of antiserum was found to correspond to a half maximal inhibition of the enzyme and presumably half combined with antibody.

On the basis of the above results it is clear that a combination between the enzyme and its specific antibody prevents a contact, or combination between the enzyme protein and its coenzyme group. On the other hand, a combination between the enzyme protein and the coenzyme inhibits from 40 to 50 per cent the combination between the enzyme protein and its specific antibody. These data could be interpreted to indicate that both the antibody and coenzyme DPN exercise combining affinities for the same groupings on the enzyme molecule. The combination between the enzyme and antibody being weakly dissociable would explain the greater degree of inhibition of enzyme activity shown when the reaction between enzyme and antibody occurs in the absence of coenzyme. Weak dissociability of the enzyme-antibody complex would likewise explain a partial inhibition of enzyme activity by the addition of an excess amount of antiserum to a reaction system which is proceeding at maximum speed.

## 5. Reports on the Inhibition of Bacterial Respiration by Specific Immune Serum

Amako (1930), Braun and Vásárhelyi (1932) studied the dehydrogenase activities of bacteria using the reduction of methylene blue (Thunberg method) as a measure. They stated that the reduction of methylene blue is appreciably inhibited in the presence of immune serum. Suranyi and Palocz (1930) measured the respiration of *B. pyocyaneus* with the Barcroft-Warburg set-up. They stated that in the presence of specific agglutinating serum, the respiration was appreciably inhibited. In the presence of immune serum and complement, following an initial increase, the respiration was completely inhibited. Wohlfeil (1933) studied the respiration of the typhoid group of bacilli in the presence of various amounts of agglutinating serum. He stated that the respiration of motile bacilli was inhibited in the presence of agglutinating serum. On the other hand, the respiration of non-motile bacilli was stated to experience an increase in the presence of agglutinating serum.

*Study of the Effect of Immune Reactions on the Metabolism of E. Typhosa and Pneumococcus.* A study was initiated by Sevag

and Miller (1948) to determine the effect of the specific sera with and without complement on the oxygen consumption by *E. typhosa* and pneumococcal cells. As is known, sensitized *E. typhosa* cells are lysed by the action of complement, and pneumococcus is resistant to this action. A quantitative method was developed to determine the weight of bacteria present in agglutinated clumps, and also the weight of bacteria which had undergone lysis during the aerobic oxidation of glucose and glycerol in the presence of immune sera and complement. This method enabled us to determine the percentage of lysis of the cells at various periods during metabolic studies.

Calculating  $QO_2$  ( $\text{mm}^3\text{O}_2/\text{mg. cells/hour}$ ) values in this manner, it was found that agglutinated *E. typhosa* (strains O-901 and H-901) and pneumococci consume volumes of oxygen equal to those of the respective controls. Intact sensitized cells with or without complement do not experience loss of oxidative activity, indicating that the formation of agglutinated clumps does not involve physical or immunological barriers to the activity of oxidative enzymes. In other words, substrates such as glucose and glycerol and oxygen can permeate between the antibody molecules deposited on the cell-walls of bacteria.

Sensitized *E. typhosa* (O-901) cells acted upon by complement undergo lysis. Immediately after lysis considerably more oxygen is used than by the controls containing the intact cells. Subsequently, the oxygen consumption of the lysed system undergoes up to 88 per cent reduction. Whether or not the previously mentioned reduction in oxygen uptake by the lysed fractions of bacilli obtained by the action of complement on sensitized cells is due to the deterioration of liberated enzyme systems or to an inhibitory effect of a specific combination between liberated intact oxidative respiratory enzymes and homologous antibodies cannot at present be answered.

In a similar study, Harris (1948) reported that oxygen uptake, with glucose as the substrate, by eight species of *Salmonella* occurred at essentially the same rate when the cells were in the presence of fresh immune rabbit serum as when in fresh normal serum. Using Thunberg technique for dehydrogenase activity, likewise the reduction of methylene blue occurred at the same ratio in both immune and normal serum.

In this connection it must be remembered that due to the large size of the antibody molecules, a combination between the oxidative

enzymes, possibly, situated inside the cell and the antibody molecules deposited on the outside wall of the cells cannot take place. This could explain the absence of inhibition of the oxidative enzymes of the bacterial cells which are agglutinated by the specific antibacterial serum.

The elucidation of these questions will require that both cell-free enzyme preparations and intact living cells be used for immunization purposes and cell-free enzyme preparations be used to determine the specific effect of immune sera prepared against such enzyme preparations.

## 6. Phosphatase and Antiphosphatase

Braun (1946) reported the result of an interesting study on the changes of blood-phosphatase levels in cows infected with *Brucella abortus*. Testing for alkaline serum phosphatase, it was found that the phosphatase level was the only one out of 23 simultaneously tested blood constituents which revealed a significant change immediately after infection. There was an average significant decrease of phosphatase levels in 39 animals showing agglutinins after infection with *Br. abortus*. In one cow there was an exceptional increase of the phosphatase level which failed to show agglutinins after infection. A second overwhelming infection of this cow with *Br. abortus* again caused a rapid increase of its phosphatase level. Six weeks later, however, this animal suddenly showed a rapidly ascending agglutination titer and the phosphatase level decreased simultaneously with the appearance of the agglutinin titer.

The enzyme phosphatase present in blood serum catalyzes the hydrolysis of monoesters of phosphoric acid, such as glycerophosphate, dihydroxyacetone phosphate, adenine nucleotide, phenylphosphate, etc. This enzyme has been reported to increase in blood serum in various diseases, such as rickets, osteogenic sarcoma, bone fractures, hyperparathyroidism, hepatitis, etc. In male guinea pigs, *Brucella* infection has been reported to affect bones, joints or other organs.

An answer to the question of the nature of factors involved in the increase in serum phosphatase in infection with *Br. abortus* must be provided for an understanding of the relationship between the decrease of phosphatase level with the rise of agglutination titer. One may

offer two possible explanations to account for the increased phosphatase level following the infection: (a) the damage inflicted on various organs by the toxic effects of the infection throwing the tissue-bound phosphatases into the blood stream; and (b) the autolysis of the infectious agent liberating bacterial phosphatases and thereby causing an increase in the serum phosphatase level of the infected host. In view of the fact that in non-infectious diseases involving damage to normal tissues and organs there is an increase in serum phosphatase level, it may seem possible that the first alternative explanation is a more plausible one. However, the amount of enzyme derived from the autolysis of an infective agent could be sufficient to affect the phosphatase level of the serum. Phosphatase elaborated in amounts comparable to those of toxins elaborated during an infection could be expected to affect the phosphatase levels of serum.

If we accept the first explanation, it is necessary to assume that the decrease of serum phosphatase level with the increasing titer of agglutinin is due to the inhibition by agglutinins of the toxic or enzyme processes of the infectious agent which are responsible for the specific damage to the tissues causing the liberation of bound phosphatases. If, on the other hand, we accept the second alternative explanation, we must consider the decrease in phosphatase level with the increase of agglutinin titer as due to the specific combination of the bacterial phosphatase with the specific antibody present in the agglutinating serum and thereby its elimination from the infected system. One may also point out the possibility that the antibody to bacterial phosphatase present in the agglutinating serum could inhibit or reduce the synthesis of phosphatase by the agglutinated bacteria. In this connection, it is interesting to note that the animals treated with living *Br. abortus* are afforded protection against abortion. Dead bacilli are found to be valueless in this respect (Topley and Wilson, 1936). Huddleson (1943) has reported that a crushed cell fraction produced active immunity in guinea pigs. This property of the active fraction was susceptible to heat and antiseptics. In this respect living bacteria and their fractions obtained from them behaved like enzymes. Any one of the above explanations would seem to require that the enzymatic activities of the immunizing agents remain intact when used for active immunization. Only under these conditions would highly effective homologous antibodies seem to form.



In an attempt to explain his own data Braun (1946) considered our view that specific antibodies formed as final reaction products in response to antigenic stimuli fulfill the function of specific inhibitors of enzymes. Applying this concept to his data he sees it necessary that the reaction: *Br. abortus* phosphatase + globulin factors  $\rightarrow$  Anti-phosphatase, take place. However, he considers it questionable whether this reaction may exist. The reason given for this reservation is that phosphatase, according to him, being a common constituent of blood is probably serologically inactive. There is no inconsistency in the application of our concept to his data if we assume that the amount of phosphatase which contributes to an increment of the serum phosphatase during an infection is derived from a bacterial source. This amount being species specifically different from that which is normally present in serum would be serologically active. The elimination of the increased amount of phosphatase concurring with the rise in titer of agglutinins could indicate that the agglutinating serum contains specific antibodies to the bacterial phosphatase. Under these conditions this bacterial phosphatase exogenous to the host system would be eliminated.

It must, however, be pointed out that the validity of one or the other of the two explanations we offered to account for the data obtained by Braun depends on the result of additional experiments. One could perhaps obtain an adequate amount of phosphatase from *Br. abortus* to prepare specific immune serum. This serum could be used to study its effect on the increased phosphatase activity of animals infected with the same organism which served as source of cell-free enzyme preparation. If the antiphosphatase serum were capable of eliminating the increment in phosphatase activity of the serum of infected animals, we could be certain of a direct relationship between a decrease of phosphatase activity and increment in agglutinin titers. Anti-serum prepared against bacterial phosphatase could be administered to animals showing increased phosphatase activity without containing agglutinins. If the serum derived from the animals thus treated showed a decrease of phosphatase activity, the same conclusion could likewise be drawn. The antiphosphatase specific serum related to *Br. abortus* would be incapable of producing a decrease in the phosphatase activity of the infected animals if the origin of the increased phosphatase is the damaged tissues or organs. For this phosphatase and that normally



present in serum are species specifically different from that derived from *Br. abortus*, and the immune serum to the latter would be inactive against the normal host phosphatases.

### C. METALLO-PROTEINS AS OXIDATION CATALYSTS AND ANTIGENS

There are a number of oxidative enzymes which contain metals such as Cu, Zn, and Mg. Chlorophyll contains Mg in a manner comparable to iron in heme. Chlorophyll combined with a specific protein constitutes the green pigment of plants. It catalyzes the transformation of carbon dioxide into carbohydrate. Zinc is a part of the enzyme carbonic anhydrase which catalyzes the reversible dissociation of carbonic acid. Copper is present in poly-phenol oxidase, known under various names. The question as to whether or not zinc and copper are combined to the protein directly or through prosthetic groups is still unsettled. Of these enzymes, only poly-phenol oxidases have been studied with respect to their antigenic property.

Gessard (1901, 1902 a, b, c; 1903, 1906) reported that tyrosinase, prepared from the larvae of crustaceans, oxidized tyrosine to a red colored product. The immune sera prepared against tyrosinase and laccase preparations exercised inhibition on the activity of the homologous enzymes. The immune serum against plant tyrosinase did not exercise inhibition on crustacean tyrosinase, but inhibited homologous tyrosinase. Gessard also reported that immune serum against peroxidase from mushrooms, *Russula delica*, inhibited the activity of homologous peroxidase but had no effect on malt peroxidase.\*

Bach and Engelhardt (1922) repeated the experiments of Gessard and confirmed his findings. Bach and Engelhardt used mushroom extract as laccase. The sera of four rabbits contained potent antibodies, the sera of two rabbits were weak, and the serum of 1 rabbit was negative.

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\*Tyrosinase catalyzes the oxidation of catechol, p-cresol and phenol in the presence of quinone, pyrogallol, dihydroxyphenylalanine, tyramine and adrenalin. Laccase catalyzes the oxidation of catechol, guaiacol, p-phenylenediamine in the presence of quinone, pyrogallol, dihydroxyphenylalanine, hydroquinone, vanillin, p-cresol and adrenalin. Peroxidase catalyzes the oxidation of the same substrates catalyzed by laccase; whereas peroxidase uses oxygen from hydrogen peroxide, laccase uses air oxygen (Graubard, 1939). For a critical discussion of the mechanism of the enzymatic oxidation of phenolic compounds the reader is referred to Nelson and Dawson (1944).

The inhibitory effect of the immune sera was tested using guaiacol as substrate. One ml. of 0.1 per cent guaiacol solution was treated with 2 ml. of buffer solution, 0.1 ml. of laccase solution and 0.1 ml. of rabbit anti-laccase serum; the final volume was made up to 10 ml. After the reaction mixture had been allowed to react for one hour, the color was measured colorimetrically. The following results were obtained:

TABLE XIX

System containing	Mg. of guaiacol oxidized			
Normal serum	0.133	0.122	0.108	0.111
Immune serum	0.059	0.052	0.062	0.058
Per cent inhibition	56	57	42	48

In another experiment, pyrogallol was used as substrate. The systems contained 0.1 g. of pyrogallol, 2 ml. of buffer solution, 0.2 ml. of serum, 0.1 ml. of laccase solution; the volume was made up to 10 ml. with water. Measurement was made after 24 hours. Purpuragallin formed was filtered, dissolved in sulfuric acid and titrated with 0.1 N potassium permanganate solution. The following results were obtained:

TABLE XX

Systems containing*	Ml. of $\text{KMnO}_4$ used		Systems containing†	Ml. of $\text{KMnO}_4$ used	
Normal serum	3.45	4.10	Normal serum	6.35	5.80
Immune serum	2.20	2.20	Immune serum	2.75	2.85
No serum	3.30	3.80	No serum	3.65	3.60
Per cent inhibition	36	46	Per cent inhibition	56	51

\*Contained buffer.

†Contained no buffer.

The authors stated that the above results were corroborated repeatedly by the data of numerous unpublished experiments. They did not find a difference in the buffering capacity of immune and normal serum. To eliminate any possibility that a difference in the physical properties of the two sera might be responsible for the in-

hibitory effect of the immune sera, they also experimented with dialyzed sera.

TABLE XXI

Systems containing	Mg. of guaiacol	Oxidized
Normal serum:	Buffered	Unbuffered
Dialyzed	0.205	0.047
Undialyzed	0.191	0.031
Immune serum:		
Dialyzed	0.078	trace
Undialyzed	0.078	trace
Without serum	0.213	0.130
Per cent inhibition:		
Dialyzed	62	90
Undialyzed	59	90

In confirmation of Gessard's observation, they found that this antibody is not destroyed by heating to 60°C. Heating at 80°C. destroyed it completely. They also observed that while the anti-laccase serum inhibited laccase strongly, normal serum accelerated its activity. In conclusion, they stated that the inhibition of laccase by homologous immune sera does not depend on the presence of any of the known laccase inhibiting factors.

Adams (1942) experimented with a preparation of tyrosinase from the domestic mushroom, *Psalliota campestris*. Its activity was 500 catechol-hydroquinone units per mg. dry weight. The copper content was 0.13 per cent of the dry weight of the enzyme. The immune rabbit serum prepared against the enzyme reacted with the antigen in 1:500,000 dilution. Normal sera were inactive. The tyrosinase from the mushroom *Lactarius piperatus* failed to show any cross precipitin reaction with the antiserum to the *Psalliota campestris* tyrosinase. Adams stated that in no case did the antibody have any effect on the catalytic activity of the enzyme in the mixture.

Duliere and Adant (1934) reported that the injection of an active tyrosinase (from mealworm) preparation into rabbits modifies the properties of the animal's serum. This serum has an inhibitory effect on a tyrosinase preparation if this is strongly active. The prepared serum, when the injected tyrosinase is very feeble or practically inactive, has no effect on an active tyrosinase.

#### D. ANTIBODY AGAINST LUCIFERASE—OXIDATIVE ENZYME OF LUMINESCENCE

Bioluminescence is an oxidative enzyme reaction. According to Harvey (1935, 1940), luminescence occurs in about forty different orders of animals and two of plants, the bacteria and fungi.

Luminescence is due to the interaction of two substances, *luciferin*, a thermostable oxidizable substance, and *luciferase*, a thermolabile enzyme, in the presence of water and oxygen. In the absence of oxygen luminescence does not occur. Luciferin is the substrate upon which luciferase exercises catalytic oxidation producing light. When oxidized by chemical agents luciferin does not give light. The oxidation of luciferin is a dehydrogenation reaction yielding oxyluciferin; the latter is again hydrogenated (reduced) forming luciferin. Luciferase also plays the rôle of a substance capable of excitation to luminesce. Luminescence results from the production of energy-rich molecules of luciferase which pick up energy from the oxidation of luciferin to oxyluciferin. Luciferase molecules can only be excited by a particular kind of luciferin. When luciferase and luciferin from two different forms having luminescence of two different colors are mixed, the animal supplying the luciferase determines the color of the resulting luminescence. This type of specificity is a universal characteristic of biocatalysts.

Luciferase is very active in dilute solution, one part in  $4 \times 10^9$  giving visible light. It accelerates the velocity of oxidation of luciferin and may be used many times, remaining practically unchanged at the end of the reaction. It is nondialyzable, destroyed by trypsin, insoluble in alcohol and in all fat solvents. It precipitates with protein precipitants.

Luciferin is a dialyzable, non-protein substance. It is soluble in alcohols and in 90 per cent acetone. In purified form it is soluble in benzene and other fat solvents. It is soluble in water, acid and alkaline solutions, and is readily oxidized in the latter solution. It is stable in water solution for many years in the absence of oxygen. Its chemical nature is unknown.

*Antibody Against Luciferase.* Harvey and Deitrick (1930) reported the production of antibody in rabbits against luciferase but

not against luciferin. The antibody was destroyed at 70°C. but not at 61°C. by heating for thirty minutes. Absence of luminescence was used as the criterion for testing the presence of anti-luciferase in the sera of rabbits immunized with a luciferase-containing extract from *Cypridina hilgendorfi*, an ostracod crustacean. The serum of a normal rabbit recently injected with luciferase produces a bright light with luciferin when tested at intervals for three to four hours. The luminescence gradually becomes less and disappears in ten hours. The luciferase is destroyed in some way in the normal rabbit. The same rabbit after receiving seven repeated injections of luciferase over a period of twenty days was tested at intervals in the same manner, and it was found that in *immune* serum the luciferase remained in the blood for less than three hours.

The antiluciferase content of the immune serum was tested as follows: The luciferase (100 mg. of material in 15 ml. of 0.8 per cent sodium chloride) was mixed with varying amounts of immune serum; the mixture was allowed to stand for 15 minutes and then 15 drops of fresh luciferin (100 mg. of material in 0.8 per cent sodium chloride) solution were added as in the following table.

TABLE XXII

Luciferase	Serum	Luciferin	Results
2 drops	Immune, 1 drop	15 drops	Good light
2 drops	Immune, 3 drops	15 drops	Faint
2 drops	Immune, 5 drops	15 drops	Very faint light
1 drop	Immune, 5 drops	15 drops	Almost no light
1 drop	Normal, 5 drops	15 drops	Good light
1 drop	Immune, 10 drops	15 drops	No light
1 drop	Normal, 10 drops	15 drops	Good light

The results show that the ability of luciferase to produce light with luciferin can be completely prevented provided enough immune serum is added. Five minutes were sufficient for complete suppression of the luminescent power of one drop of luciferase mixed with eight drops of immune serum.

The rabbits immunized with luciferase solution, which also contained oxyluciferin, showed no unequivocal evidence of having developed an anti-luciferin.





## *Part VI*

# Physiology and Biochemistry of Shock

A VAST LITERATURE has accumulated concerning investigations of the various aspects of shock. It is beyond the scope of this monograph to survey every phase of the problems which have been investigated. In the present discussion, subjects dealing with and related to the pathology of the antigen-antibody reaction in the animal system will briefly be considered. The general literature has been adequately reviewed by Feldberg (1941), Dragstedt (1941, 1945); Dragstedt and Wells (1944), Rocha e Silva (1944), Selle (1946), and Rose (1947). Anaphylaxis, caused by an antigen-antibody reaction, is a specific biochemical problem in immunology. The pharmacological actions of peptone, trypsin (papain and ficin) and animal venoms are likewise biochemical in nature. The toxic reactions produced by these chemically unrelated primary factors, however, would seem to possess certain common features. It is claimed that the liberation of histamine, as a toxic reaction product, appears to be one of the important factors in nearly all of these phenomena. Its release as a factor in trypsin shock has, however, been recently contested (Wells, Morris and Dragstedt, 1946; Tagnon, 1945). Danielopolu (1947), however, claims that acetylcholine is the primary and histamine a secondary factor in anaphylaxis (see below).

It has also been variously pointed out that the physiological changes accompanying shocks caused by anaphylaxis, peptone, trypsin and animal venoms are similar to those accompanying traumatic shock. A survey of the similarities among these reactions might, therefore, be advantageous for an understanding of these related phenomena.

### 1. A Description of Anaphylactic Shock

Anaphylaxis is considered as a special and severe form of a more general pathological process, namely, of allergy. Allergic diseases of

man, such as hay fever, pollen asthma, atopic urticaria, and perhaps others are based upon similar mechanisms. In all these cases, antibodies are formed which unite with the antigen and in each case it is the union of antigen and specific antibody followed by cell injury which produces the allergic symptoms.

It is generally accepted that the antigen unites with antibody which is fixed to cells. Exemplified by anaphylaxis, explosive and violent in character, with marked effects on the respiratory, circulatory, glandular and smooth muscle systems, this reaction is a fundamental and challenging biochemical problem in immunology whose specific mechanism awaits elucidation. Undoubtedly cell injury occurs during the antigen-antibody reaction. As a consequence, or associated with the antigen-antibody reaction, in addition to histamine, several physiologically active substances, such as heparin, possibly choline or acetyl-choline, potassium, glucose and various enzymes are let loose. Asphyxia associated with all types of shock plays an important role in cell-injury and cellular disorganization. In connection with the view that it is not the free or circulating antibodies, but those attached to the fixed tissue cells which are most concerned with this reaction, Moon (1938) cited the following: (a) the incubation of antibody with antigen *in vitro* does not produce a potent injurious product; (b) antigen-antibody precipitate is relatively innocuous; (c) antibody and antigen simultaneously injected fail to produce shock in nonsensitized animals; (d) in passive sensitization an interval of several hours must elapse before the injection of antigen produces shock symptoms; (e) if the blood from a sensitized animal is replaced by blood from normal animals of the same species, the animal still remained sensitive; and (f) organs whose vessels had been washed clean of blood by perfusion with salt solution still responded characteristically to the antigen.

The dominant symptoms of bronchospasm in the guinea pig, of circulatory failure in the dog, and cardiocirculatory failure in the rabbit could all be harmonized on the basis of a spastic contraction of strategically located smooth muscle. The critical site of activity for this reaction is the smooth muscle in the bronchioles of the guinea pig, the hepatic veins of the dog, and the pulmonary arterioles of the rabbit. These considerations demonstrate that the fundamental reaction in all animals is probably identical (Simonds, 1919; Dragstedt, 1941). Sufficient histamine is claimed to be liberated during

fatal guinea pig anaphylaxis to account for the death of the animal.

**a. Symptoms in Guinea Pigs.** After intravenous injection into a sensitized animal, there is a prodromal period of a few minutes during which the animal usually sneezes, scratches its nose, becomes restless, discharges urine and feces, becomes weaker and rolls over on its side. Acute respiratory difficulty then develops, and death from asphyxia may follow in less than 10 minutes. Due to a swiftly developing stenosis of the bronchioles there is inadequate air exchange with a resulting asphyxia despite the violent respiratory efforts. This bronchiole reaction is due to a direct action on the muscular walls of the bronchi and not due to a central or reflex nervous reaction. An initial moderate rise in systemic blood pressure followed by a gradual fall, is similar to that occurring in asphyxia from any cause. It is considered that the anaphylactic reaction is primarily pulmonary and the circulatory effects are secondary in importance (Dragstedt, 1941). The anaphylactic symptoms described here have been found to be associated with the liberation of histamine from the lungs of the guinea pig. An increase in the blood histamine of anaphylactic animals up to 13 times the normal values has been found (Code, 1939; 1944).

**b. Symptoms in the Dog.** The anaphylactic reaction is associated with dyspnea, vomiting, salivation, general weakness, diarrhea and a marked progressive fall in blood pressure due to peripheral vasomotor paralysis. With the decrease in arterial pressure there is a decrease in the blood volume of the kidney, intestine and spleen, while there is a large accumulation of blood in the liver. The vascular reaction in anaphylactic dogs has been accounted for by the amount of histamine released from the liver (Dragstedt and Mead, 1936a, 1936b); and the severity of the shock in dogs is accompanied and paralleled by a reduction in liver histamine (Ojers, Holmes and Dragstedt, 1941).

The loss of coagulability of dog's blood during anaphylaxis has been found to be due to the simultaneous liberation of heparin with the histamine from the injured liver (Jacques and Waters, 1941).

**c. Symptoms in the Rabbit.** The anaphylactic shock produced by intravenous reinjection of antigen causes quickening of respiration, sinking of the animal upon its abdomen, expelling of feces and urine, fleeting hyperemia followed by anemia of the ears; the heart beat becomes feeble, agonal convulsions may develop and the animal may die within a few minutes.

Acute anaphylactic death in the rabbit is due to a spasmodic constriction of the pulmonary artery, followed by rapid dilatation of the right side of the heart which is the most characteristic abnormality in this animal (Coca, 1919).

Various observations have indicated that there exists a consistent similarity between the circulatory reactions in the anaphylactic rabbit and the pharmacological effects of histamine (Dale and Laidlaw, 1910-11; Rocha e Silva, 1940). Katz (1940) observed that when antigen is added to the blood of a sensitized rabbit *in vitro*, there is a striking shift of histamine from corpuscular elements to the plasma. This observation has been confirmed (Dragstedt, Ramirez and Lawton, 1940; Rose and Browne, 1941).<sup>\*</sup> The relation of these observations to anaphylaxis in the rabbit has been critically discussed by Rose (1947) who would seem to agree with Dragstedt (1945) that the fundamental phenomenon in rabbit anaphylaxis is a liberation of histamine from cells into the plasma.

In any species, additional symptoms in anaphylaxis are aggregation of polymorphonuclear cells in the capillaries of the lungs to produce a leucopenia, a decrease in coagulability of the blood, most marked in the dog, a decrease in the amount of complement in the blood, and a fall in the body temperature. In anaphylaxis the following chemical changes have been observed. There is loss of histamine from cells into the plasma, histamine-like substances are liberated, serum potassium increases, plasma suffers lowering of carbon dioxide combining, or alkali reserve capacity, or there is an immediate and progressive acidosis. Acidosis appears before the onset of recognizable clinical symptoms of shock. There is also an increased secretion of urinary creatine, uric acid and urea ammonia, indicating derangement in liver function and protein metabolism. These changes will be further discussed below in relation to the biochemical mechanism of shock.

## 2. Peptone Shock

The shock produced by the injection of peptone resembles true anaphylactic shock in many respects. Contraction of the bronchioles in the guinea pig, fall of blood pressure and congestion of the liver in

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<sup>\*</sup>See, however, discussion on page 438.



the dog, loss of coagulability and multiple small subserous hemorrhages are features common to both types of shocks (Biedl and Kraus, 1909; Feldberg and O'Connor, 1937; Dragstedt and Mead, 1937). In the intact dog, administration of peptone causes a release of histamine into the blood, a decrease in liver histamine and a marked thrombocytopenia (Holmes, Ojers and Dragstedt, 1941). Gotzl and Dragstedt (1942) reported the liberation of histamine from rabbit white cells *in vitro* on addition of peptone, and *in vivo*, peptone caused a fall in whole blood histamine and thrombocytopenia. Jacques and Waters (1941) observed that heparin is produced in peptone shock. In peptone and anaphylactic shock, the occurrence of a short period of hypercoagulability, agglutination and the ensuing disappearance from the blood of the platelets which contain most of the histamine, suggested to Quick (1942) the concept that, in either type of shock, the sudden liberation of histamine causes the agglutination of platelets. The agglutinated masses of platelets are carried to the capillary beds of the liver, lung and spleen and other tissues where disintegration begins and thromboplastin and histamine are liberated. The former substance is responsible for the short period of observed hypercoagulability, and the latter for certain of the various symptoms observed in these shocks.

In peptone shock there is a marked decrease in the volume of oxygen consumed by the animals, and a simultaneous increase in the lactic acid content of blood. The relation of these changes to the mechanism of shock will be discussed below.

### 3. Shocks Caused by Proteolytic Enzymes

a. **Trypsin.** The pharmacological action of proteolytic enzymes, particularly of trypsin, has been variously studied. The findings of Dragstedt and Wells (1944) can be summarized in the following paragraphs. In unanesthetized dogs, the intravenous injection of trypsin results in vomiting, urination, defecation and collapse, comparable to that occurring in anaphylaxis. In anesthetized dogs, the injection of trypsin results in prompt and profound fall in systemic blood pressure, with little or no change in pulmonary artery pressure, congestion of the liver and a corresponding congestion of the viscera. Injection of a given amount of trypsin into a branch of the portal vein in the dog was followed by a much more conspicuous and lasting fall of carotid

blood-pressure than that observed following a similar injection into the femoral vein.

In *rabbits*, intravenous injection of trypsin results in a momentary rise and more conspicuous fall in systemic blood pressure, a marked rise in pulmonary arterial pressure, a marked leucopenia, and a partial to marked incoagulability of the blood. With increased amounts of trypsin injected intracutaneously a local necrosis comparable to that of the Arthus reaction is produced.

With *guinea pigs*, the intravenous injection of trypsin is rapidly fatal. On opening the chest the lungs collapse and show areas of local hemorrhage. The gross appearance indicates that death is due to an embolic phenomenon, and to an asphyxia resulting from broncho-stenosis.

In *cats*, the intravenous injection of trypsin results in a fall in the systemic blood pressure and a decreased clotting of the blood.

**b. Papain and Ficin.** Rocha e Silva (1943) reported that ficin and papain are toxic upon intravenous injection. Molitor, *et al.* (1941) reported the results of a study on the toxicity of ficin in mice, rats, guinea pigs, rabbits, cats, and dogs. They found that sublethal intravenous administration of ficin produced vomiting, bloody diarrhea, and general prostration. Autopsy showed severe irritation of the gastro-intestinal tract ranging from inflammatory reactions to erosions. Small doses of ficin reduced the erythrocyte count and markedly prolonged the blood clotting time. Parenteral injection of ficin caused severe tissue damage.

**c. Chymotrypsin.** The effects of chymotrypsin have been reported to be less potent than trypsin although they are qualitatively similar (Rocha e Silva, 1943).

Tagnon, *et al.* (1945) compared the symptoms of shocks produced by trypsin and chymotrypsin. They pointed out that trypsin brings about intravascular clotting when it is injected intravenously and, therefore, it is difficult to evaluate in a given experiment what changes result from the clotting activity and what changes result from the proteolytic activity of trypsin, since clotting agents such as thrombin, and proteolytic agents such as trypsin, both produce shock in animals (Tagnon, 1945). Because of the fact that trypsin brings about intra-

vascular clotting and shortens the clotting time, while on the other hand, chymotrypsin prolongs the clotting time and does not clot blood, and also plasma contains a trypsin inhibitor and there is no inhibitor for chymotrypsin in serum, the differences in the effects of these two enzymes were investigated. They found that a dose of 40 to 60 mg. of chymotrypsin/kg. was lethal to dogs. At necropsy, no intravascular blood-clots were found by microscopic examination. In contrast, a dose of 10 to 50 mg. of trypsin/kg. was toxic for dogs. Post-mortem examination (2 mg. of trypsin/kg.) showed intravascular blood-clots. Absence of blood-clotting or the prolongation of clotting time by chymotrypsin was attributed to the possible digestion of fibrinogen and prothrombin and thereby to their reduction in amounts, and to possible modification of the physiological properties of the fibrinogen and prothrombin of the plasma.

Tagnon, *et al.* (1945), found that the initial effect on blood pressure by chymotrypsin was much less than the rapid and marked fall in the blood pressure produced by trypsin.

d. **The Question of the Proteolytic Liberation of Histamine as Cause of Trypsin Shock.** Emphasizing the similarities observed in anaphylactic shock, and shock caused by histamine and proteolytic enzymes, Rocha e Silva (1940a, 1941) reported demonstrating the liberation of histamine (and other catabolic products) by the administration of proteolytic enzymes to animals. Contraction was produced by the action of trypsin on mammalian smooth muscles (intestine and uterus of guinea pig, intestines of rabbit, cat, dog and mouse, and virgin uterus of rat). Intravenous injection of trypsin produced effects resulting in the collapse and death of rabbits, dogs and cats. In cats and rabbits the obstruction of the pulmonary circulation was deemed to be responsible for tryptic shock.

In experiments on perfusion of guinea pig lungs it was demonstrated (Rocha e Silva, 1940b) that trypsin was capable of liberating histamine from the tissues, and it was suggested that this may offer a basis for understanding the pharmacological effects of trypsin. It was also reported (Ramirez, *et al.*, 1940) that the injection of trypsin in dogs is followed by an increase of the histamine-content of the blood plasma due to the liberation from the liver of 6 to 10 mg. of histamine for the

whole organ. A similar amount of histamine is reported to be discharged from the liver of dogs following anaphylactic shock (Ojers, *et al.*, 1941). Rocha e Silva and Andrade (1943) reported results indicating the liberation of histamine from rabbit blood cells by the action of papain.

However, the hypothesis that trypsin shock is a consequence of tryptic liberation of histamine has recently been questioned. Wells, *et al.* (1946) reinvestigated the question of the toxicity of trypsin in relation to histamine liberation and arrived at the following conclusions. Guinea pigs dying from the intravenous injection of trypsin fail to show either the rise in blood histamine or the emphysematous lungs characteristic of the liberation and action of histamine. In these experiments, benadryl (beta-dimethylaminoethylbenzhydrol etherhydrochloride), which is a potent antagonist to either administered or liberated histamine, afforded neither the guinea pig nor the dog any significant protection against the lethal action of intravenously injected trypsin. In other experiments, they found that benadryl is much less effective in reducing the blood pressure response to trypsin than it is in reducing the response to histamine. Determining the blood histamine before and during tryptic shock in the dog they concluded that in none of these animals was the blood histamine of sufficient magnitude to explain the severity of the reaction produced. Only in one dog was a rise in blood histamine observed. They reported that the amount of histamine released during the perfusion of dog's liver is very small. And the liberation of histamine from isolated guinea pig lungs by perfusion with trypsin solutions is deemed to be of no consequence to the toxicity of trypsin in the intact animal in view of the absence of any pulmonary effects of histamine when trypsin is injected into the intact animal.

The effective prevention of the action of trypsin on isolated smooth muscle by arginine as due to an antagonism of arginine to liberated histamine is questioned. They suggest that the trypsin itself may be antagonized by arginine. They reject the idea that the positive "trypan-blue reaction" produced by trypsin is an indication of the liberation and action of histamine. In view, also, of the findings of Tagnon (1945) that intravascular clotting by trypsin injected intravenously may play a significant role in the elaboration of the toxic symptoms noted, the histamine hypothesis, relative to the toxicity of trypsin, is considered doubtful.



#### 4. A Summary of Some of the Physiological Changes Accompanying Shock

Bronchostenosis and asphyxia, labored breathing, a slow fall in blood pressure, hemorrhages, intestinal congestion (guinea pig), fall of blood pressure, congestion of liver and the corresponding congestion of viscera (dog) are some of the common symptoms of anaphylaxis, peptone and trypsin shock. Intravenous injection (dog) of trypsin produces blood-clotting by converting prothrombin to thrombin before an excess of heparin is released preventing clotting. In anaphylaxis and peptone shock, the formation of a blood-clot does not occur.

In dogs, vomiting, salivation, diarrhea, urination and collapse are common to both anaphylaxis and trypsin shock. In rabbits, peptone and anaphylactic shock cause thrombocytopenia; trypsin shock causes leucopenia; and anaphylaxis produces either the aggregation of polymorphonuclear cells in the capillaries of the lungs, or a pneumonia. Anaphylaxis causes spasmodic constriction of the pulmonary artery followed by rapid dilatation of the right side of the heart and acute heart failure, and in trypsin shock a marked rise in pulmonary artery pressure occurs.

In anaphylaxis and peptone shock there is a loss of histamine from the liver to the plasma; in shock caused by trypsin liberation of histamine has been claimed and contested. The principal chemical changes in various shocks are characterized by the liberation of choline-like substances, an increase of serum potassium, a decrease in serum alkali reserve, an increase of lactic acid in the blood, a decrease in the consumption of oxygen, and an increase in the secretion of urinary creatine, uric acid and urea ammonia.

#### 5. Histamine Shock

**a. Effects of Histamine.** The close resemblance between the pharmacological effects of histamine in animals and those of anaphylactic shock was pointed out by Dale and Laidlaw (1910). And Dale (1929) proposed the theory that histamine is liberated from the tissues of animals consequent to the interaction of antigen with antibody. This theory of Dale has been supported by subsequent investigations.



Histamine has been reported to exercise three main pharmacological actions: 1. An intense capillary dilator effect on the circulatory system. 2. An excitation of involuntary muscles of smooth type of most organs. 3. A stimulation of secretory systems—salivary, gastric, pancreatic, intestinal and lacrimal.

A prominent pharmacological action of histamine is its spasmogenic action on smooth muscle of the bronchioles, gastro-intestinal tract, uterus, ureters and gall bladder. Histamine indirectly induces arteriolar constriction or dilatation depending on the animal species or the locality of the vascular bed in a given species. It causes dilatation of the capillaries and venules, and thus causes hyperemia and skin wheals in humans. The production of increased capillary dilatation and greater capillary permeability is attributed to the action of histamine. However, these effects also result from anoxia. The latter effect is involved in the production of localized edema. Histamine is a potent stimulator of the lymphatic flow.

In guinea pigs, an intravenous injection of 0.3 to 0.5 mg. of histamine per kg. guinea pig causes death by inducing spasm of the bronchial muscle and suffocation. In rabbits, the administration of histamine causes transient hypertension and then hypotension. The death of the animal is due to circulatory failure which is secondary to a marked constriction of pulmonary blood vessels. In cats, dogs, and man, the blood pressure falls rapidly upon the administration of histamine. Dogs are killed by 1 to 3 mg. of histamine per kg. Man also is sensitive to milligram quantities of histamine. The mouse, rat, and frog, on the other hand, are very resistant to histamine, and the toxicity of histamine is manifest only after receiving doses of 500 mg. or more.

**b. Distribution of Histamine.** Histamine has been isolated from intestinal mucosa (Barger and Dale, 1911), and detected in a variety of tissues (Abel and Kubota, 1919). It has been isolated in crystalline form from animal tissues (Best, *et al.*, 1927) and whole rabbit blood (Code and Ing, 1937). Considering blood as the source of histamine, the largest amount has been found in the rabbit and smallest in the cat. In the mouse over 60 per cent of the histamine is found in the skin. The tissues of the central nervous system are virtually devoid of histamine, but histamine is found in large amounts in the peripheral sensory nerves which are the only nerves capable of antidromic stimulation.

Seventy to 90 per cent of the blood histamine is held in an inactive

state within the white blood elements; the small remainder circulates in a free state in the plasma or is held within the red blood corpuscles. In the so-called "shock" tissues as the lungs of the guinea pig and the liver of the dog, predominant amounts of histamine are present. Here, likewise, histamine, in the normal animal, is held in an inactive state, but is assumed to constitute a rich and ready source for toxic action.

Halpern (1945) reported that a very high level of histamine and histamine-like substances has been found during attacks of asthma and in the blood, urine and sputum of allergic patients. Related to this, asthmatic victims are found to be extremely sensitive to histamine. In urticaria and Quincke's edema, the histamine has often been found to be very high in the blood. In certain sick headaches and during painful crises of gastroduodenal ulcer a high level of histamine has been found. Nevertheless, Halpern concludes his discussion in the following manner. "However, we think that the study of histamine cannot itself furnish us with decisive clues. *We think that the action of histamine confines itself to strictly local reactions appearing at the very level of the tissues or the organ which constitutes what is called the 'shock organ.'* Generally speaking, histamine is destined to be formed to die on the spot. It is only exceptionally that histamine *spreads throughout the circulatory system*. The measurement of circulating histamine, therefore, is only a relative value and as long as we do not know the *local manifestations, that is to say the tissue modifications of histamine*, we can formulate more or less vague hypotheses only."

In connection with the above stated destruction of histamine at the site of its liberation the observations of Granroth and Nilzen (1948) are of interest. They demonstrated that extracts prepared from the skins of guinea pigs, rabbits, cats and human subjects exercise high histaminolytic activity. [For the destruction of histamine in various mammalian tissues see Zeller (1942), and for a discussion of data in support or opposed to the histamine theory of allergy and anaphylaxis, see Ratner (1943)].

**c. Origin of Histamine.** Tissue histamine might be considered as a decarboxylation product of the naturally occurring l-histidine. Werle and Krautzun (1938) reported the formation of histamine from the decarboxylation of l-histidine by the kidney and liver tissues of guinea pig, rabbit, hamster and mouse; a trace of decarboxylase was observed in the pancreas of guinea pig, rabbit and hamster. It is interesting to

note that the enzyme l-histidine decarboxylase is inhibited by adrenaline and arterenol (Werle, 1942) which exercise antihistamine effects. Holtz, *et al.* (1938) reported the presence of l-histidine decarboxylase in the wall of the small intestine, but not that of the colon and rectum, of the guinea pig. Since the fetal guinea pig intestine also formed histamine from l-histidine, the formation of histamine in the intestinal wall of the adult animal is most likely not due to bacterial activity (Holtz, Credner, and Reinhold, 1939). These observations strongly suggest the presence of an l-histidine decarboxylase in mammalian tissues. The amounts of histamine formed, in *in vitro* experiments are small. It has not been isolated, but rather characterized pharmacologically. On the isolated guinea pig small intestine, the extracts caused contraction of the muscle which was not abolished by atropine; and when the muscle had become specifically insensitive to histamine by its administration in large doses the extracts were found to be inactive (Werle and Hermann, 1937). This effect is considered typical for histamine (Barsoum and Gaddum, 1935).

The question of an exclusive source of total histamine in the animal system cannot be answered. Both the l-histidine decarboxylases of animal tissues and of intestinal bacteria may be contributory in this respect. Histamine can readily form from l-histidine by the action of micro-organisms normally present in the intestinal tract. This mechanism could account for the formation of histamine in the intestine. Ackermann (1910) reported that putrefactive bacteria could produce histamine from histidine by a process of decarboxylation. And Melanby and Twort (1912) observed that normal organisms of the intestinal tract of man and animals could likewise produce histamine, and that the latter is a regular constituent of the intestinal content and feces. Gale (1946) reported that *E. coli* and related organisms, nine of 10 strains of *Cl. welchii* type A, strains of *Cl. welchii* types B, C and D, and one of two strains of *Cl. fallax* possessed histidine decarboxylases. He studied *E. coli* amino acid decarboxylases extensively. It is therefore reasonable to suggest that histamine formed by intestinal bacteria could readily diffuse through the intestinal wall and be stored in various tissues, in a manner similar to the storage and utilization by the animal system of essential vitamins synthesized by intestinal flora.

d. **The Function of Antihistamine Substances and Their Pharmacological Action.** The release of histamine during anaphylaxis would naturally produce symptoms which would, in certain respects, be common to both anaphylactic and histamine shock. This is further emphasized by the fact that the drugs which counteract or prevent the action of histamine are also effective in anaphylaxis. Experimentally, an antihistamine substance inhibits the physiological action of histamine on the isolated intestine and uterus of animals, on the bronchi, on the vessels; it protects the animal against lethal histamine shock. The only action it does not suppress is the excito-secretory power of histamine on the gastric, pancreatic and salivary secretions. Used therapeutically, the antihistamines have already given remarkable results in several allergic states, mainly serum sickness, some urticaria, hay fever, etc. (Halpern, 1945; Mayer, 1947; Loew, 1947). The prophylactic administration of Antergan (2339 RP) has been found (Halpern, 1945) to be sufficient to avert completely anaphylactic shock: blood pressure hardly changes, the usual hemoconcentration is absent; dyspnea, algidity, hypothermia and sphincteral troubles are likewise absent. This striking protective action is not permanent. After a delay of about 5 days, these animals are again sensitive. It is then possible to produce just as severe an anaphylactic shock in them as one produces in the test animals.

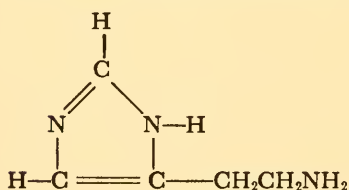
The antihistaminic drugs do not prevent the release of, or destroy the released histamine. They prevent histamine from exercising its action on the sensitive sites. The manner by which the antihistamines bring about this effect is still unknown. Gruhzit and Fiske (1946) found that benadryl and A-446 administered orally, intravenously, subcutaneously and intraperitoneally were toxic in albino mice and rats, rabbits and dogs. Both substances caused a complex syndrome of excitement reactions predominantly neurogenic in origin involving motor, sensory and automatic nervous systems. Barbiturates controlled excitant neurologic reactions, but did not prevent respiratory-cardiac depression. Irrespective of the mode of administration, toxic doses of either compound caused excitement, spastic ataxia, extreme irritability, sensitivity to sound, mydriasis which is due to a disorder of the central nervous system, painful hyperesthesia, convulsive attacks, and respiratory and myocardial embarrassment. Death occurred from respiratory



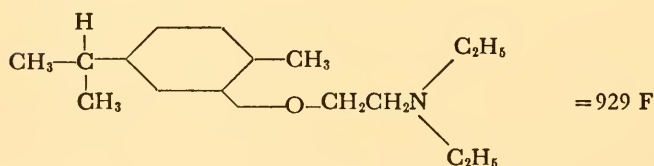
and myocardial depression following violent excitement and terminal prostration.

Epinephrine is another substance which has long been known to produce effects which are opposite that of histamine. Injection of histamine will stimulate the release of epinephrine from the medulla of the adrenal gland (Feldberg, 1941). On the other hand, the intravenous injection of epinephrine in man will produce an increase of the histamine content of the blood plasma (Staub, 1946). Epinephrine and its congeners, however, are not considered as antihistamine drugs (Rose, 1947) since they induce prominent effects such as bronchodilation, vasoconstriction, decreased capillary permeability, inhibition of intestinal activity which present the antitheses of those produced by histamine. Atropine and other antispasmodic drugs, and certain amino acids and derivatives of histamine are capable of antagonizing some of the effects of histamine. However, Rose considers that these compounds are non-specific since their ability to counteract, or relax smooth muscle and to antagonize acetylcholine, barium and spasmogenic agents equals or exceeds their effectiveness in opposing histamine. Those drugs which are potent-histamine antagonists are said to act as blocking agents with some degree of specificity as regards antihistamine action. Pharmacologically, the specificity is considered relative and not absolute.

#### e. Histamine and Antihistaminic Substances



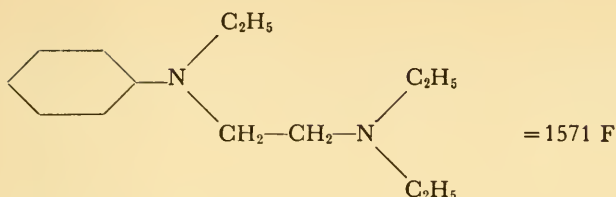
Histamine



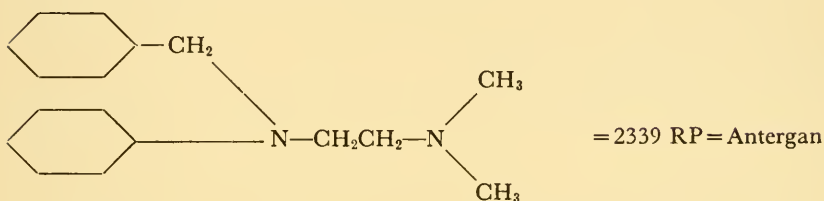
= 929 F

2-Isopropyl-5-methylphenoxyethyldiethylamine

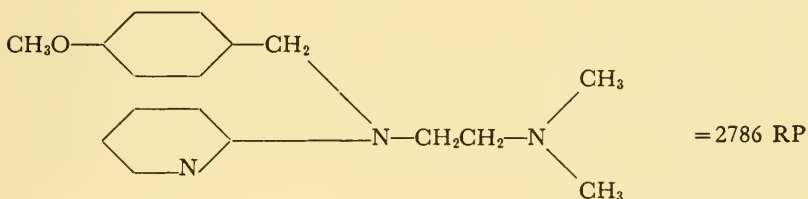




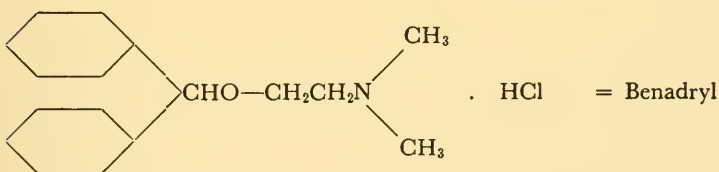
N'-phenyl-N'-ethyl-N-dimethylethylenediamine



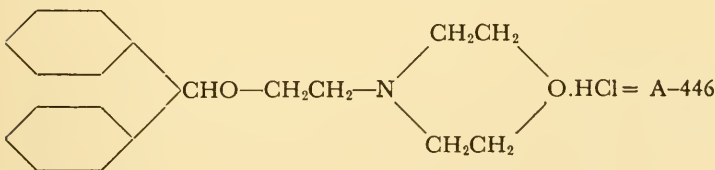
N'-phenyl-N'-benzyl-N-dimethylethylenediamine



N-p-methoxybenzyl-N-dimethoaminoethyl-α-aminopyridine



β-Dimethylaminoethyl-benzhydryl-ether-hydrochloride



β-Morpholino-ethyl-benzhydryl-ether-hydrochloride

f. **A Comparison of the Syndromes of Histamine and Anaphylactic Shock.** Several physiological changes which occur in anaphylaxis and histamine shock are commonly shared. In the guinea pig, in both cases, there is the spasm of the bronchial smooth muscle, causing suffocation and death, spasm of the smooth muscle of the gastrointestinal tract, uterus, ureters and gall bladder. In the dog, the muscular walls of the efferent hepatic veins constrict, producing localization of the blood in the capillary spaces of the liver. In the guinea pig in both cases, there is capillary dilatation and capillary permeability causing localized edema.

Another similarity between histamine and anaphylactic shock is the protection of the animals against the action of both by antihistamine drugs.

A characteristic feature of anaphylactic shock is the failure of the formation of blood-clot. This failure is absent in histamine shock. Another difference between the two phenomena is that the severity and persistence of the distention of the dog's liver with lymph and blood in anaphylaxis is far greater than that occasioned by histamine shock. These differences speak against the identity of the underlying mechanism in the two instances, though the characteristic similarities of certain syndromes suggest that certain factors are common to the two phenomena.

## 6. Metabolic Changes in Shock

In fully developed shock there is a diminution in the circulating blood volume and a cutting off of the supply of oxygen and nutrients to the tissue. It is observed that there is first an increase in blood sugar; it then decreases reaching a state of hypoglycemia, especially in the terminal stages of shock. This may be due to depletion of liver glycogen in rats in tourniquet shock, in rabbits in gravity shock, and in hemorrhagic shock, and to a marked increase in the utilization of carbohydrate by the peripheral tissues (Wilhelmi, 1948). Depletion of glycogen in shock is accompanied by increased carbohydrate utilization, increase in blood lactate and pyruvate and a striking rise in the ratio of lactate to pyruvate and a shift toward anaerobic metabolism in the tissues. Outpouring of lactate from the tissues may be correlated with a marked fall in arterial blood plasma carbon dioxide and blood pH.

According to Dubois-Ferriere (1945), trauma to the hind limbs of rabbits under conditions of venous occlusion leads to a fall of blood pressure. The blood shows an increase in adenosine triphosphate, acetylcholine, and potassium ion derived from the damaged tissue.

The most significant change in blood chemistry during shock is an increase in plasma amino nitrogen, which is related to the failure of the liver to assimilate amino acids for protein and oxidative metabolism. During the development of shock catabolic changes in the tissues with respect to inorganic phosphate, pentose, glucose, amino acid nitrogen and lactic and pyruvic acid have been found (McShan, *et al.*, 1945). During the anoxia of hemorrhagic hypotension in dogs there is a destruction of liver enzymes and coenzymes, in particular, destruction of cocarboxylase (dephosphorylation), cozymase, alloxazine adenine dinucleotide and an inactivation of the protein moieties of amino acid oxidase and lactic dehydrogenase (Greig and Govier, 1943; De Turk and Greig, 1945; Alexander, 1944). Le Page (1946a, 1946b) reported that the tissues of animals in shock exhibit greatly elevated inorganic phosphate and lactic acid, depleted glycogen, adenosine triphosphate and phosphocreatine, and possess an abnormal accumulation of phosphopyruvic acid. Of the tissues examined brain, heart, muscle, kidney and liver, in the terminal stages of Noble-Collip, tourniquet, and hemorrhagic shock, the liver showed the most complete depletion of the stores of adenosine triphosphate compounds and other sources of readily available energy. In the biochemical sense, complete exhaustion of energy reservoirs—adenosine polyphosphates—means the death of tissue (Le Page, 1946b). McShan, *et al.* (1945) and Meyer, *et al.* (1946) hypothesize that the essential feature of fatal shock is the critical exhaustion of the supplies of readily available energy, in the form of phosphocreatine and adenosine triphosphate.

## 7. Acetylcholine in Anaphylactic Shock

Various investigators have reported that anaphylactic shock is accompanied by the liberation of acetylcholine (Went and Lissak, 1936; Farber *et al.*, 1944). Danielopolu (1947a) claims to have demonstrated that the liberation of acetylcholine is the direct cause of anaphylactic shock and that histamine plays a secondary role. It may

therefore be worthwhile to discuss certain properties of acetylcholine before consideration of its role in anaphylaxis.

**a. Destruction and Synthesis of Acetylcholine. Cholinesterase.** Acetylcholine esterase is the enzyme which destroys acetylcholine (Loewi and Navratil, 1926). According to Nachmansohn, *et al.* (1948), a highly purified enzyme preparation containing 1 mg. of protein was capable of hydrolyzing 60 g. of acetylcholine in one hour.

The enzyme is found in nerve and muscle. They are the only tissues in which this specific enzyme has been found, whereas all other tissues contain distinctly different types of esterase (Nachmansohn, *et al.*, 1947). Other tissues and serum contain enzymes capable of hydrolyzing acetylcholine, often in high concentration, but apparently of slightly different specificity than the muscle or nerve esterase.

**Choline Acetylase.** This enzyme mediates the synthesis of acetylcholine from choline and acetate. The essential role of acetylcholine in the conduction of the impulse in the muscle fiber as that in the nerve fiber necessitates that its synthesis through an enzyme in these tissues be insured. The synthesis of acetylcholine by isolated brain (Quastel, *et al.*, 1936), and in nerve endings (MacIntosh, 1941) has been reported. Nachmansohn, *et al.* (1947) demonstrated the presence of choline acetylase in extracts from pigeon breast muscle, skeletal muscle of guinea pig, and cardiac muscle of rabbit and guinea pig. The test-systems contained choline, acetate, eserine, potassium, magnesium, cysteine, adenosine triphosphate and coenzyme (probably a pantothenic acid derivative, Lipmann, *et al.* 1947). Choline acetylase was reported to be completely absent in liver and kidney extracts similarly prepared and tested. In these reactions *eserine* serves as the inhibitor of choline esterase to permit the preservation of synthesized acetylcholine. *Cysteine* serves to maintain the -SH groups of the enzyme choline acetylase. *Potassium* is necessary for the activity of the enzyme. *Coenzyme* which probably contains a pantothenic acid derivative is necessary for the acetylation of choline. Acetate is converted to acetylphosphate by receiving an energy rich phosphate from adenosine triphosphate. Acetylphosphate donates its acetyl group to choline.

**b. "Muscarinic" and "Nicotinic" Actions of Acetylcholine.** Acetylcholine has two types of action in the body. Resembling closely the action of the alkaloid *muscarine*, acetylcholine acts at or beyond the

postganglionic nerve endings of the parasympathetic nervous system producing dilation of blood vessels, increased tone of bronchioles and gut, slowing of the heart, and increased glandular secretion; the use of the term *muscarinic* action is related to these actions of acetylcholine.

Like the alkaloid nicotine, acetylcholine acts on all the autonomic ganglia, both sympathetic and parasympathetic and on the skeletal myoneural junction. It stimulates these when used in small doses and paralyzes them when large doses are used. These effects of acetylcholine on autonomic ganglia and on voluntary myoneural junctions is called *nicotinic*.

**c. Antagonists to Acetylcholine.** Ergotamine, nicotine, curare, and atropine are antagonists of acetylcholine. *Atropine* blocks all the muscarinic action of acetylcholine whether they are excitatory as in the intestine, or inhibitory as in the heart. *Nicotine* and *curare* block only the nicotinic action of acetylcholine, nicotine acting especially on the ganglia and curare on striated muscle. In a system where both striated and smooth muscles are present (i.e., intestine of the fench, a primitive fish, see Goodman and Gilman, 1941), the application of both atropine and curare obviates the contraction of the organ; atropine specifically blocks the action of acetylcholine on the smooth muscle, and curare specifically blocks that on the striated muscle of the system.

Curare, although having the same points of attack as acetylcholine, has only a paralyzing effect. It acts at the neuromuscular junction, that is, it prevents the end-plate from responding normally. If curare is eliminated—excretion, washing, etc.—the neuromuscular transmission is re-established.

*Pilocarpine* is believed to act highly selectively on structures supplied by postganglionic cholinergic nerves. In this respect, pilocarpine shows the muscarinic and not the nicotinic effects of acetylcholine. Muscular and glandular responses occur to muscarine and pilocarpine after complete nerve degeneration. Similarly, evidence from frog single fibers suggests that even in denervated muscles, the end-plate region remains especially sensitive to depolarization and stimulation by acetylcholine (Kuffler, 1946).

**d. The Role of Acetylcholine on the Effector Organs.** Acetylcholine by its muscarinic action and cholinergic impulses which release acetylcholine have been known to produce constriction of bronchial muscle, and secretion of bronchial glands; increase of the secretion and



motility and tone of stomach and intestine, and cause the relaxation of their sphincters; contraction of the urinary bladder and relaxation of the trigone and sphincter; and increased secretion of salivary and lachrymal glands. A very important action of acetylcholine as of cardiac vagal impulses is the slowing of the heart rate or the production of auriculo-ventricular (A-V) block. This is accomplished by a depression of the sino-auricular (S-A) node which initiates the heart beat or auriculo-ventricular node which transmits the impulse from auricle to ventricle. These are reactions which are accentuated in anaphylactic shock.

e. **Effects of Administered Acetylcholine in Man.** A study dealing with the *autonomic*, *sensory* and *motor* responses in man to injections of acetylcholine has been reported (Harvey, Lilienthal and Talbot, 1941). Immediately after injection into the brachial artery the arm below the elbow became flushed, warm and sweated profusely. These responses persisted for 10 to 15 minutes then slowly waned. The injection was followed immediately by excruciating pain coursing down the forearm into the palm and fingers. The character of the pain was variously described as "burning," "tearing," or "tingling." Simultaneously with the development of pain in the injected arm the grip power of the fingers and hand was greatly reduced so that only the most feeble and partial flexion of the fingers could be effected voluntarily. There was complete recovery of normal motor power 30 to 60 seconds after injection. There was no constant synchronized movements in the fingers or hand following the injection of acetylcholine. In one subject, there developed a brief movement of the fingers and hand resembling the carpal spasm of tetany. In four subjects, there appeared in the relaxed palmar and forearm muscles brief localized twitches of insufficient tension to produce more than a slight movement of the fingers.

The motor phenomena of paresis and fasciculation occurred only transiently and feebly with the doses of acetylcholine which were employed. With prostigmine, however, these effects were much more prolonged and profound. It was believed that the prostigmine paresis results from the inhibition of cholinesterase which permits the concentration of acetylcholine to rise to a paralyzing level.

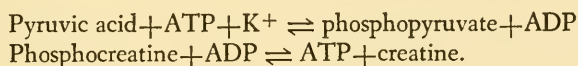
The observed effects of intra-arterially injected acetylcholine in man upon the sweat glands and the peripheral vascular bed was believed

to agree with the present concept of the functions of cholinergic nerves. The diffuse and intense pain may have resulted from generalized stimulation of nerve endings subserving pain.

**f. *In Vitro* Action of Acetylcholine on Muscle Contraction.** It has been reported that the proximate intra-arterial injection of minute amounts (2  $\mu$ g) of acetylcholine into mammalian skeletal muscle produces a powerful twitch equal in tension to that induced by a single maximal motor nerve volley (Brown, Dale and Feldberg, 1936). According to Brown (1937, discussed by Nachmansohn, 1945) the amount of acetylcholine necessary to produce a maximal twitch of striated muscle is 100,000 times as high as that liberated per nerve stimulus. It may also be noted that the concentration of cholinesterase at the motor end-plates is many thousand fold higher than in the whole muscle, which would mean that the rate of hydrolysis of acetylcholine at motor end-plates is equally greater than in the whole muscle.

Cantoni and Eastman (1946) reported that the administration of histamine (1:100 million), acetylcholine (1:10 million), pilocarpine (1:100,000), barium chloride and acetyl- $\beta$ -methylcholine was followed by temporary depression of the contractile responsiveness of the intestinal strip of the guinea pig. On the other hand, a maximal contraction in response to large doses of potassium chloride did not result in a decreased contractility of the preparation. In fact, a small increase in the K/Ca ratio of the perfusion fluid was sufficient to neutralize the effect of large doses of histamine, acetylcholine, pilocarpine, and barium chloride.

Potassium ion is essential for the phosphorylation of pyruvic acid in the presence of an adequate concentration of adenosine triphosphate (ATP). The formation of phosphopyruvate is a necessary step for the synthesis of (a) glycogen, and (b) phosphocreatine. In the synthesis of phosphocreatine, phosphopyruvate immediately transfers its phosphate to creatine,  $\text{ATP} \rightleftharpoons \text{ADP}$  (adenosinediphosphate) acting as intermediate link.



In muscle contraction, the breakdown of ATP is believed to be the most probable immediate source of energy. According to Buchthal, *et al.* (1946) creatine phosphate and acetylphosphate have no effect

on striated muscle; ATP initiates contraction and changes birefringence when applied in minute amounts to isolated striated muscle fibers of the frog, thus establishing the breakdown of ATP as the reaction nearest in time to contraction. On the other hand, the breakdown of phosphocreatine is believed to produce during nerve activity sufficient energy for the electric action potential. It has been calculated that a single nerve impulse requires only 1/50,000 of the energy of a muscle twitch (A. V. Hill, cited by Nachmansohn, 1945).

In *in vitro* studies, Jordan and Oster (1948) reported that in potassium chloride solution ATP induces the coiling of actomyosin. They consider the ATP-actomyosin interplay of fundamental importance in muscular contraction. The function of potassium in this reaction is, likewise, of critical significance.

The observation of Cantoni and Eastman (1946) that histamine and acetylcholine, etc. depress the contractile responsiveness of the intestinal strip of guinea pig may suggest that these agents are interfering with the above mentioned critical roles of potassium in the metabolism of muscle. This interference is overcome or antagonized by an increase in the concentration of potassium ion in the systems studied. In this connection, reference may be made to the possibly related observation of Rocha e Silva and Beraldo (1948). They reported that potassium ion accelerates the antihistaminic action of pyribenzamine, neo-antergan, antistin, benadryl, etc. on the guinea pig ileum suspended in tyrode solution. Magnesium ion, on the contrary, had a strong decelerating effect; calcium ion in general exercised a predominantly depressing effect; strontium ion, even when present in the low concentration of 0.0025 molar, had a definite accelerating effect on the antihistaminic action of the drugs.

Buchthal and his associates have studied the interaction of acetylcholine and ATP on striated and smooth muscles of mammals. ATP or ADP in amounts of  $10^{-5}$   $\mu$ g, applied to the curarised or non-curarised isolated muscle of the frog produces tetanus-like contractions which are accompanied by action potentials. Also, in curarised and non-curarised striated and smooth muscles of mammals, contractions are released when the substance is applied by close arterial injection (Buchthal, 1947). Intra-arterial injection of acetylcholine decreases the electrical excitability and changes the strength-duration curve of muscle for a considerable time after its application. Previous application of

ATP does not affect the strength-duration curve of a muscle. In denervated muscle intra-arterial injection of acetylcholine renders the muscle insensitive to subsequent application of ATP, and prevents the release of contraction by ATP (Buchthal, *et al.*, 1946, 1948).

ATP applied to muscle increases its sensitivity towards intra-arterially injected acetylcholine four to ten-fold. Inorganic triphosphate has a similar effect. The effect is not on the neuromuscular transmission but on the muscular substance, since it is elicited also in curarized muscle. Why small amounts of acetylcholine prevent ATP administered through the artery from producing a response is not clear. Large amounts also inhibit the release of contraction by intramuscularly applied ATP. Since, however, the electrical excitability was retained, it was assumed that acetylcholine somehow prevents a contact between ATP and the contractile muscle elements (Buchthal and Folkow, 1948).

**g. Role of Acetylcholine in Anaphylactic Shock.** A consideration of the above presented data shows clearly the various effects acetylcholine is able to exercise. It is obvious that a certain concentration of endogenous acetylcholine must be built up for a measurable effect. The question of whether or not a physiologically significant concentration of this substance can be built up at the site of "shock organs" must be considered. The high activity of choline esterase in destroying the liberated acetylcholine must form part of our consideration. Apparently there are other esterases, besides choline esterase, which destroy acetylcholine. It is reported that  $10\mu\text{g}$  of acetylcholine incubated for one hour with dog serum plus a minute amount of eserine, when injected into a cat, manifests the characteristic vasodepressor action; without eserine this action was not observed. On the other hand, the analogues of acetylcholine which are resistant to the hydrolytic action of serum esterases, in the absence of eserine, are found to elicit a fall in blood pressure. Carbaminocholine,  $(\text{CH}_3)_3\text{N}(\text{OH})\text{-CH}_2\text{CH}_2\text{O-CONH}_2$ , and acetyl- $\beta$ -methylcholine,  $(\text{CH}_3)_3\text{N}(\text{OH})\text{CH}_2\text{CH}_2\text{-(CH}_3\text{)OCOCH}_3$ , are of these types. The latter compound is approximately 200 times more potent than acetylcholine,  $(\text{CH}_3)_3\text{N}(\text{OH})\text{-CH}_2\text{CH}_2\text{OCOCH}_3$ , in evoking cardiovascular responses in man. Choline, though 500 to 100,000 times less potent, resembles acetylcholine in acting as vasodepressor agent (Goodman and Gilman, 1941).



However, in evaluating the possible concentration of acetylcholine which may accumulate in the shock organs the tremendous difference in the concentration of cholinesterase present in the motor end-plates and in the mammalian muscle should be taken into account. It has been calculated that in the muscle the time of hydrolysis of acetylcholine is 40,000 times longer than in the end-plates (Nachmansohn, 1945). Due to this great difference, the accumulation of a toxic amount of acetylcholine in the muscle during or preceding a shock may not appear to be an impossibility.

**h. Liberation of Acetylcholine in Anaphylactic Shock.** Went and Lissak (1936) reported that choline-like substances are liberated during an anaphylactic shock from the heart muscle. The perfused heart of the sensitized guinea pig showed slowing and rhythmless reaction when antigen was added to the perfusion fluid. This effect was prevented by the perfusion of the heart with Tyrode's solution containing atropine. This effect was not produced by histamine. In the heart perfusate of a guinea pig surviving anaphylactic shock a much larger amount of choline was found than in normal heart muscle. The choline contents of the heart muscles of normal, sensitized and desensitized animals (the heart muscle of surviving guinea pigs) were alike. In contrast, the heart of the shocked animal contained a lesser amount of choline. On the other hand, the histamine content of all the three types of heart muscle remained the same. Acetylation of the perfusion fluid obtained in these experiments yielded a substance possessing properties comparable to those of acetylcholine in its action on the frog heart and on the leech. Farber, *et al.* (1944) reported the liberation of a noteworthy amount of acetylcholine in the shocked heart and none in extracts of normal heart. There was no difference in the amounts of acetylcholine found in the shocked and normal lungs and intestines of guinea pigs.

It has been reported that the antihistaminic drug 933F prevents the action of epinephrine, acetylcholine and histamine. The concentration of 933F required were in the following order:

Histamine > acetylcholine > epinephrine

Antergan (2399RP) was found to have no action on adrenalin, but inhibited acetylcholine and histamine action on the intestine and pregnant uterus of the guinea pig. It has a hypotensive effect itself



and does not prevent the action of acetylcholine and histamine on the blood pressure and circulation, but does so in conjunction with atropine. On this basis, the suggestion is made that therapeutically in anaphylactic shock both should be given, since, after atropinization, acetylcholine and histamine cause adrenalin secretion from the adrenals (Danielopolu, Popesco, and Mezinesco, 1941-1945).

According to Danielopolu (1947a, 1947b, 1947c), liberation of acetylcholine is the primary factor of anaphylaxis, and the production of histamine is a secondary factor; there is no histamine production without acetylcholinogenesis. The phenomenon of anaphylactic shock is identical with that provoked by an excitation of the parasympathetic system (which reacts by a liberation of acetylcholine): *inhibition of the heart by auriculo-ventricular (A-V) block, hypotension, hypermobility of digestive and vesical systems and uterus, and leucopenia involving mononucleosis and eosinophilia*. Intravenous injection of acetylcholine produces the above named symptoms resembling (without being identical) those produced by anaphylactic shock. Intratracheal injection of acetylcholine into guinea pigs produces a respiratory syndrome similar to anaphylaxis: *expiratory dyspnea, bronchial râles, asphyxia*, etc. *Autopsy reveals pulmonary lesions identical with those which he found in anaphylactic shock*.

A simple mechanical excitation of the tissue provokes the phenomenon of acetylcholinogenesis. Intravenous injection of antigens and injection of a suspension of gelatin liberate acetylcholine. Eserine and strophanthin favor anaphylaxis by inhibiting cholinesterase, whereby a greater concentration of acetylcholine in the tissues accumulates. Atropine in higher doses blocks anaphylaxis by blocking the cells of the terminal organs. Adrenalin in small doses favors anaphylaxis and blocks it in large doses. A hyperconcentration of acetylcholine and, secondarily, of adrenalin precursor accompany anaphylaxis.

In this connection it must be pointed out that Loewi and Navratil (1926) showed decisively that inhibition of the heart resulting from vagal stimulation is due to the liberation of acetylcholine. It was also demonstrated that the heart muscle contained choline-esterase which rapidly hydrolyzed acetylcholine after its liberation, limiting the duration of its action. The action of this esterase was inhibited by prostigmine (eserine).

i. **Experiments Cited to Support the Acetylcholine Theory of Anaphylaxis.** Antigen provokes general vasodepression in sensitized cats and dogs; it causes the vessels of the paw of dog to dilate; stops the frog's heart; and augments the tonus of the intestine and uterus of the guinea pig and rabbit. Eserine and strophanthin inhibit the action of cholinesterase, permitting the accumulation of acetylcholine. In this manner these inhibitor drugs exaggerate the above effect provoked by antigen.

A suspension of agar stimulates contraction of the isolated uterus and intestine. This effect is identical with that of acetylcholine. When the organ is eserinated the effect is accentuated. Atropine inhibits this effect. The isolated heart of the guinea pig is stopped if suspended in agar solution. Atropine inhibits this effect on the heart. This blockage is not due to potassium ion or histamine; these are still able to produce their effects on atropinized tissue.

In rabbits, the intravenous injection of 6 ml. of a gelatin solution causes death within two to three minutes. This shock is inhibited by atropine. The injection of 3 ml. of gelatin solution does not produce shock in the rabbit. However, the animal which is subcutaneously eserinated (0.25 to 0.5 mg. of eserine) succumbs to shock within 2 minutes when 2 to 3 ml. of gelatin solution are injected intravenously. This shock is due to the liberation of acetylcholine.

An amount of antigen-antibody complex prepared *in vitro* which does not produce shock in the guinea pig, produces fatal shock in animals eserinated subcutaneously. It is to be noted that eserine promotes the shock produced by injection of acetylcholine, or an antigen, or a gelatin solution. Atropine inhibits anaphylaxis even in eserinated guinea pigs.

It is found that strophanthine favors anaphylactic shock because it inhibits cholinesterase and thereby permits the accumulation of acetylcholine. A dose of an antigen-antibody mixture which is incapable of provoking shock in normal guinea pigs causes fatal shock in strophanthinized guinea pigs. But, if the tissue is rendered refractory to acetylcholine by means of atropine, the same antigen-antibody mixture fails to provoke shock in the strophanthinized and eserinated guinea pigs.

A large dose of atropine inhibits anaphylactic shock. It is claimed to exercise two principal actions: (a) it inhibits the activity of choline-

terase; and (b) it modifies the cells of the terminal organs, rendering them refractory to acetylcholine (parasympathofrenatric action). These two effects are intensified by increasing the dose of atropine. A small dose which inhibits choline esterase (anti-acetylcholinolytic) favors the action of acetylcholine. A higher dose of atropine, though anti-acetylcholinolytic, renders the cells of the terminal organs refractory to acetylcholine. It thus makes it necessary that a greater dose of atropine be used to protect the animal from the action of acetylcholine, and anaphylactic shock. In the rabbit, it is necessary to use enormous amounts of atropine to prevent shock, because the tissue of rabbits contains an enzyme which inactivates atropine by splitting it into tropine and tropic acid.

In the guinea pig, toxin-antitoxin mixture is known to fail to produce shock. After the eserization of the animal, the same mixture provokes a classical acetylcholine type of anaphylactic shock. Two successive injections of eserine (0.025 mg. and 0.1 mg./injection) were subcutaneously administered to a guinea pig. After a period of eight minutes, the intravenous injection of toxin-antitoxin complex produced a non-fatal anaphylactic shock. When 0.5 mg. of eserine/injection was administered twice, after a period of eight minutes the intravenous injection of 1 ml. of toxin-antitoxin mixture into the jugular vein caused immediate shock and death in five minutes. In this series of tests eighteen guinea pigs were used.

In another set of experiments, the intestine of the rabbit, which is very sensitive to acetylcholine, immersed into Tyrode's solution containing a mixture of diphtheria toxin-antitoxin mixture, showed marked tonus. After the shock, the bath was replaced with a fresh Tyrode solution and a new mixture of toxin and antitoxin was added; a lesser degree of contraction of the intestine was observed. A third test with fresh materials similarly made eliminated the response of the same intestine, indicating the exhaustion of acetylcholine in the intestine. However, when acetylcholine was added to a fresh bath containing toxin-antitoxin complex, the deacetylcholinized intestine responded as in the first test. Histamine also plays a role in the production of anaphylactic shock, but it is claimed that there is no histamine production without the production of acetylcholine.

Epinephrine has been shown to have an important influence upon the nervous system within the spinal cord, and on synaptic trans-

mission. It may also stimulate, or inhibit, intestinal activity; it can increase, or decrease, blood pressure. But it is generally believed that it brings out sympathomimetic effects.

Danielopolu believes that epinephrine causes elevation in blood pressure, vasodepressor effects, increase in metabolic rates, etc. in effector cells. This response unlocks a compensating *parasympathetic* cellular response. In treatment with ergotamine, and antihistaminic 883F, which inhibit sympathomimetic action, the action of adrenalin is rendered exclusively parasympathomimetic. He claims that the response in the latter case is produced as a result of the liberation of acetylcholine. However, he is unique in attributing the inhibitory effect of epinephrine to liberation of acetylcholine. Cannon has demonstrated two chemical components in sympathetic activity (sympathin E and sympathin I). But Cannon never identified the latter with acetylcholine (Goodman and Gilman, 1941). It would follow from Danielopolu's reasoning that the action of epinephrine treatment, preceded by a treatment with ergotamine, and 883F, should promote the phenomenon of shock. He reported that the shock caused by gelatin, which is also a shock caused by the liberation of acetylcholine, is equally promoted by epinephrine following a treatment with ergotamine.

Experimenting with the virgin uterus of the guinea pig, treatment with acetylcholine, potassium ion and histamine caused increased tonus of the uterus. In the early phase of pregnancy the response of the uterus to these agents was similar to the above. In an advanced phase of pregnancy adrenalin exercised an inhibitory effect, and acetylcholine, potassium ion and histamine were excitatory. It was demonstrated that atropine, which paralyzes the guinea pig intestine, excites the uterus. But, while atropine inhibited the action of acetylcholine on the uterus, it had no action on the potassium and histamine effects. In this manner he believed that he had a precise method to demonstrate whether a shock is caused by acetylcholine, histamine or potassium ion.

j. **Summary of Evidence in Favor of Acetylcholine Theory.** The following summary of evidence is submitted in support of the Acetylcholine Theory of Anaphylaxis. The hyperconcentration of acetylcholine and, secondarily, of adrenalin precursors in anaphylaxis results in hyperfunction of all the organs where the parasympathetic is excitatory



and the sympathetic inhibitory. This is accompanied by the hyperproduction of histamine which results in further excitation. The affirmation of this hypothesis Danielopolu bases on the following: (a) hypotension and hypermobility of certain smooth muscle organs is induced by histamine, similarly to acetylcholine; (b) in anaphylaxis these symptoms are inhibited by atropine which acts only on acetylcholine; (c) eserine and strophanthin, which do not affect the action of histamine, favor shock; (d) the convulsions which appear during anaphylaxis can only be explained by the action of acetylcholine, since they are not produced by histamine, this is also true of the inhibition of the heart; (e) acidosis accompanies anaphylaxis, while in shock due to histamine there is an alkalosis. He further pointed out that during histamine shock there is a *greater decrease in serum-albumin* than in serum-globulin, while during anaphylaxis there is an increase of serum-globulin, and a decrease of serum-albumin. Starvation inhibits anaphylaxis but does not affect histamine shock. Injection of glucose inhibits anaphylaxis but has no effect on histamine shock. The clotting of the blood is not changed in histamine shock but is greatly decreased in anaphylaxis.

The above described observations of Danielopolu derived from experiments involving the use of eserine, etc. may very well permit the accumulation of acetylcholine and thereby be responsible for the symptoms described. However, the claimed relationship of these observations to anaphylaxis occurring under normal conditions may be fortuitous.

## 8. Metabolic Changes in Anaphylactic Shock

**a. Liberation of Potassium.** Schittenhelm, *et al.* (1927, 1928) studied the potassium and calcium content of the blood of various anaphylactic animals, particularly the dog and rabbit. Sensitized rabbits immediately after the reinjection of antigen experienced convulsion and expulsion of feces, etc. The blood of the carotid artery, portal and hepatic veins drawn within two minutes showed the following (Table XXIII) results in rabbits.

In the shock organs the potassium values showed marked decrease (Schittenhelm, Erhardt and Warnat, 1928). The changes in dogs were similar to those of rabbits.



TABLE XXIII

Rabbits	Carotid artery			Portal vein			Hepatic vein		
	K	Ca	K/Ca	K	Ca	K/Ca	K	Ca	K/Ca
Anaphylactic	mg%	mg%	—	mg%	mg%	—	mg%	mg%	—
Normal	105.1	13.4	8.4	122.1	14.6	8.4	124.6	14.3	8.4
(average value)	22.4	12.6	1.8	27.9	12.7	2.2	25.9	12.0	2.15

High plasma potassium values have also been reported in various allergies, and bronchial asthma, dermatitis and severe infections (Fenn, 1940). Thaler (1935) reported that the injection of histamine causes an increase in the plasma potassium.

An increase in both potassium and acetylcholine has been detected in the venous blood from stimulated muscle, and in the heart after vagal stimulation. It is reported that the injection of potassium liberates both acetylcholine and adrenalin from the adrenal gland.

Asphyxia is one of the conditions produced in anaphylactic shock. Eppinger, *et al.* (1928) reported that during histamine and peptone shocks there is marked decrease in the volume of oxygen consumed by the shock animals and a simultaneous increase in the lactic acid content of the blood. In asphyxia, there is a liberation of potassium and a stimulation of the adrenals. When the nerve fibre, which is exceptionally rich in this ion, is stimulated or deprived of oxygen, potassium diffuses rapidly into the surrounding fluid. The potassium is restored again after the re-admission of oxygen. It is believed that a nerve adequately supplied with oxygen does not lose potassium; a potential difference between the surface of the fibre and its interior is maintained by a difference in  $K^+$  concentration. The excitability of the nerve depends on the maintenance of this condition. In asphyxia when potassium diffuses out into the surrounding fluid a reduction in the potential difference and therefore a corresponding reduction in the excitability of the nerve occurs. Depending on the concentration of the diffused potassium in the surrounding fluid, a complete loss of excitability and therefore muscular function, would result.

**b. Potassium-Calcium Antagonism.** Besredka (1907), and Kastle, *et al.* (1913) reported that calcium chloride, injected the day before

the administration of the second dose of antigen into sensitized guinea pigs, protects the animals against anaphylactic shock. The effect of potassium chloride in liberating adrenalin is antagonized by calcium chloride; however, calcium chloride itself is reported to liberate adrenalin (Katz and Katz, 1937). A synergism between calcium and adrenalin is reported (Fenn, 1940) to exist. This synergistic effect may, therefore, be of significance in explaining the protection afforded to anaphylactic animals by calcium and epinephrine, the latter liberated by the action of the former.

Calcium appears to play a part in decreasing the permeability of the cell membrane and the irritability of cells in general. In calcium deficiency in higher animals a condition of hyperirritability develops through an interference with the neuromuscular mechanism. Calcium in excess, or in normal concentration, but in the absence of potassium, lengthens systole at the expense of diastole, finally resulting in *calcium rigor*. Potassium acts in a reverse manner if in excess or unbalanced by calcium. Gradually the cardiac cycle becomes diastolic, and heart ultimately comes to rest in the completely relaxed state. Thus, for the normal beat of the heart, calcium increases contractility and prolongs systole, and potassium, having a reverse effect, reduces contractility and favors relaxation. These relationships may play a significant role in the inhibition of the heart in anaphylactic reactions.

c. **Acidosis in Anaphylactic, Histamine, and Peptone Shock.** Eggstein (1921), and Hirsch and Williams (1922) reported that acute anaphylactic shock in dogs is associated with an immediate (within two to three minutes following the injection of antigen) and progressive acidosis. The acidosis appears before the onset of recognizable clinical symptoms of shock. When the carbon dioxide combining capacity of the blood plasma falls below 25 volumes per cent the animal usually dies. In a later study, Eggstein (1924) found also that the alkali reserve of the blood plasma is greatly decreased in shocks produced by the intravenous injection of toxic *proteoses* and typhoid vaccines in dogs and in human cases. A definite relationship between the decrease in the alkali reserve of the plasma and the lowered blood pressure in *toxemia* was noted. The animal's life was in danger when the alkali reserve of the blood fell below 30 volumes per cent.

Eppinger, *et al.* (1928) determined muscle lactic acid and the

volume of oxygen consumed before, during and in the post-shock period of shocks produced by histamine and peptone. They obtained the following table of results:

TABLE XXIV

Exp.	Periods of Observation	Lactic Acid in Muscle
		mg%
I	Before shock	167
	Histamine shock, 10 min. period	269
	After shock, 10 min.	221
	“ “ 20 min.	213
	“ “ 30 min.	170
	“ “ 40 min.	157
II	Before shock	180
	Shock (histamine)	240
	After shock, 10 min.	220
	“ “ 30 min.	190
	Shock and muscular work	340
	After 10 min.	300
	“ 30 min.	280

The results show that histamine (and peptone) shock causes an increase in the lactic acid content of the muscle. When associated with muscular work, shock caused a greater accumulation of lactic acid.

The measurement of oxygen consumption and carbon dioxide evolution during histamine and peptone shock showed a decrease of oxygen consumption, and an accumulation of lactic acid in the muscles of the shocked animals. (Danielopolu reported that there is acidosis in anaphylactic shock, and alkalosis in histamine shock.) This effect was due to a circulatory disturbance. Following the shock period, the surviving animals consumed more oxygen than they did before the shock. When the crisis was over and normal conditions were restored, the oxygen consumption resumed the normal rate. The increased oxygen consumption following the shock period was apparently due to the oxidation of accumulated lactic acid and other products.

**d. Metabolic Factors in Anoxia.** In connection with the above cited increase in muscle lactic acid, it is of interest to recall the reference previously made with regard to the increased urinary creatine, uric acid and urea ammonia (Miller, 1940) in anaphylactic shock. In

asphyxia, which is a prominent symptom of shock, there is a decrease in the energy-rich adenosine-triphosphate. Increased lactic acid formation in the muscle indicates that the supply of oxygen is inadequate due to the prevalence of anaerobic conditions. Increase in creatine secretion in the urine indicates that the transfer of high energy phosphate from adenosine-tri- or di-phosphate to creatine and from creatine phosphate to adenosine-diphosphate has suffered a set-back. The breakdown of adenosine-triphosphate (ATP) is the immediate source of contraction energy.

The muscle contains relatively limited amounts of ATP. Due to the fact that adenosine-diphosphate formed in the above reaction can be rephosphorylated at the expense of phosphocreatine, danger of the exhaustion of ATP does not exist. Under prolonged or sustained activity a newer source of ATP is provided by glycolysis. For every hexose unit of glycogen metabolized four molecules of ATP are produced. Of these three are a net gain, which can be drawn upon for increased muscular activity.

If the normal, unpoisoned muscle is allowed to work *anaerobically*, the following are the factors involved: ATP remains unchanged; phosphocreatine disappears yielding free creatine and inorganic phosphate; glycogen disappears yielding lactic acid. Anaerobic recovery permits the resynthesis of phosphocreatine from creatine and inorganic phosphate, and here again with the disappearance of glycogen lactic acid is formed (Baldwin, 1947). Under anaerobic conditions the metabolism of glycogen to pyruvate provides the means for the formation of lactic acid by the reoxidation of the reduced cozymase I. Without the reoxidation of the reduced cozymase by pyruvate, no further phosphoglyceric acid could be formed and therefore the generation of ATP would come to an end. However, from the standpoint of muscular work the anaerobic metabolism is very inefficient; potential energy is locked in lactic acid molecules.

Under aerobic conditions, the reduced cozymase I is rapidly oxidized through the flavoprotein-cytochrome-cytochrome oxidase system and no, or a very small trace of, lactic is produced. If the rate of muscular exertion and therefore the rate of glycogen breakdown increases, cozymase is reduced proportionately more readily, requiring therefore an equal pace of oxygen supply. When the oxygen supply fails to keep pace with the demand for the reoxidation of the reduced cozymase,

lactic acid is accumulated which rapidly escapes from the muscle into the blood and then to the liver for transformation into glycogen (Engelhardt, 1946; Lundsgaard, 1930; Baldwin, 1947; Szent-Györgyi, 1947, 1948).

In the types of shock discussed here, the most prominent reaction seems to be asphyxiation resulting from an acute bronchial constriction. This reaction alone would produce immobilization of the energy sources. This can account for the observed metabolic changes—decrease in alkali reserve of blood plasma and increased lactic acid formation; decrease in ATP, increase in urinary creatine; failure to deposit glycogen or increased glycolysis, etc.—in severe anaphylactic or other shocks. However, the nature of the factors arising from the combination of antigen and antibody, leading to bronchial constriction and asphyxia, the block of the heart, convulsion, etc. is the critical question which requires clarification before an understanding of the basic mechanism of these shocks.

## 9. Theories on the Liberation and the Role of Proteinases in Anaphylactic Shock

Of the most critical factors claimed to be responsible for the phenomenon of shock, histamine and acetylcholine have already been considered. There remains for consideration the role of proteolytic enzymes which are claimed to be liberated as a result of injury to tissue cells subsequent to or associated with the combination of an antigen with antibody fixed in cells. It is postulated that the liberation of histamine is mediated by tissue proteolytic enzymes, and since these enzymes must first be liberated by an antigen-antibody reaction, the ideas about the manner by which this liberation can occur must be considered. In this connection it may be noted that the liberation of acetylcholine does not require a proteolytic action. A simple stimulus applied to nerve tissue is believed to cause the liberation of acetylcholine. This liberation can occur within a millisecond. On the other hand, gauged by the speed of *in vitro* activity of proteolytic enzymes, it has been a concern that proteolytic action *in vivo* cannot proceed fast enough to liberate a sufficient amount of histamine and therefore cannot account for anaphylactic reactions occurring with dramatic suddenness.



The liberation and the role of proteolytic enzyme before or during anaphylactic shock, or any kind of shock, as a cause of these processes has been claimed and refuted for over four decades. It has been oft claimed that shocks are preceded by the liberation of proteases which produce peptone-like digestion products which, as discussed above, produce characteristic anaphylactic symptoms. The fact, however, that the reinjection of microgram quantities of an antigen into a sensitized animal is capable of producing almost an immediate shock has been advanced as an argument against the "proteolytic enzyme" mechanism of anaphylactic shock. It is argued that a few minutes' time interval preceding shocks of sudden and explosive nature is too short a period to liberate proteases capable of producing an adequate amount of toxic peptone-like digestion products. It is difficult to state at present to what extent one can place sufficient weight on such arguments. We are in the dark concerning the relative speed and quantitative relationship between the proteolytic processes occurring *in vivo* and *in vitro*. Almost nothing is known about the possible speed of chemical reactions within the organism and of the mechanisms involved.

One may perhaps make reference to an observation by Jacobson (1947). He reported two hitherto unrecognized chymotrypsins, one of which was from 2 to 2.5 fold, and the other 1.5 fold more active than the ordinary chymotrypsin. It is not at all impossible that in animal systems the native proteolytic enzymes are far more active than they are in an artificial environment. In connection with the role of proteolytic enzymes in shock, it may be of interest to refer to the studies of Jobling and Petersen (1914a, 1914b, 1914c). After a review of older findings concerning the inhibitory role of lipids on the proteolytic activity of serum proteases, they reported that antitrypsin activity of serum is due to the presence of compounds of unsaturated fatty acids. The sera from which these protective lipids have been removed by various means were rendered toxic for the homologous animal. The toxicity is due to (a) an alteration in the mechanism of clotting, with resulting intravascular clotting; (b) the exposure of the native serum proteins; and (c) the formation of toxic split products (primary proteases) by digestion. The return of the extracted lipids neutralizes the toxicity of sera. The toxicity of the extracted serum is the result of the serum protein digestion products rapidly produced by serum proteases freed from the inhibiting action of serum lipids. Anaphyla-

toxins represent sera rendered toxic by partial removal of serum antitrypsin (lipids).

Dead bacteria treated with serum became more resistant to the proteolytic action of serum due to the adsorption of antitryptic lipids from serum. Bacteria previously treated with serum or with oils do not adsorb serum antitrypsin.

Fresh guinea pig serum was mixed with typhoid bacilli, and placed in the ice chest overnight (the serum so treated showed marked decrease in the antitryptic value). A portion of the mixture was centrifuged until it became clear, and was then injected intravenously into guinea pigs to determine its toxicity. Two ml. of clear supernatant caused immediate death of a guinea pig weighing 210 grams, with all the evidences of anaphylaxis. Repeated experiments yielded similar results. This effect was claimed not to be due to the action of a toxin.

Bronfenbrenner summarized (1944) the results of several of his studies on the mechanism of anaphylaxis. He also believed that an antigen-antibody reaction lowers the normal antitryptic titer of serum yielding activated serum "trypsin." Tryptic action liberates heparin preventing the clotting of the blood. Activated "trypsin" likewise produces polypeptides and peptones. Combined action of activated trypsin, and of polypeptides and peptones results in injury to tissue and the liberation of histamine. As Jobling and Petersen (1914) reported, Bronfenbrenner (1944, for earlier references) had found that serum properly adsorbed with kaolin or starch *in vitro* also activates serum ferments, with subsequent autodigestion of the serum, which produces anaphylaxis-like symptoms when injected intravenously, especially in homologous animals.

The above observations are significant in relation to the shock produced by proteolytic enzymes. Since the blood histamine is in blood cells and cell-free serum most likely is free from histamine, it does not appear that the anaphylactic shock produced by sera treated in the above manner could contain sufficient histamine to be responsible for shock in the guinea pig. It may, therefore, be related to the action of proteolytic factors.

## 10. The Question of Proteolysis and Release of Histamine in Shock Produced by Proteolytic Enzymes and in Anaphylaxis

A number of investigators have pointed out a parallelism between the effects of an antigen in a previously sensitized animal and those of trypsin in similar animals without the necessity of sensitization. These effects, release of histamine and heparin from the tissues by the injection of trypsin, the phenomenon of desensitization, the inhibition of smooth muscle contraction by arginine and histidine are considered restricted to anaphylactic experiments and significant. As in anaphylactic shock, proteolytic enzymes produce: (a) failure of blood to clot, perhaps due to release of heparin; (b) less conspicuous, but nevertheless significant, is the brief period of increased clotting which precedes the failure of blood to clot, and which may be due to the conversion of prothrombin to thrombin. When trypsin is injected into the intact animal it is believed there is sufficient time for such an effect to occur before the tissue release of heparin obscures the accelerated clotting (Rocha e Silva, 1943). Tagnon, *et al.* (1945) found intravascular clots by post-mortem examinations of dogs after the injection of 2 mg. of trypsin/kg. Mirsky and Freis (1944) reported a similar finding.

Dragstedt and Wells (1944) reported a parallelism between the pharmacological activity and enzyme activity of trypsin. The inactivation of trypsin by acid, heat, and serum inhibitor produced an equal decrease in proteolytic potency and pharmacological activity. As they pointed out, however, the pharmacological effects are not the consequence of gross or obvious digestion of either blood or tissues. Intravenous injection of trypsin produces its dramatic effects upon the circulation within fifteen to twenty seconds. The contraction of an isolated intestinal strip occurs within one to three seconds. The liberation of histamine from rabbit blood cells to plasma occurs promptly and before any morphological evidence of digestion is present. It may be pointed out, however, that our chemical methods of *in vitro* quantitative measurement may be too crude to permit the measurement of proteolytic effects associated with the above processes. Mirsky and Freis (1944) report that intraperitoneal injection of trypsin causes extensive

tissue damage. Microscopic examination of the kidneys of both rats and rabbits revealed cloudy swelling and vascular congestion, degeneration of the cells of the collecting and distal convoluted tubules, massive deposition of eosinophilic granular and fibrillar material in the tubules and glomerular spaces, and focal collections of lymphocytes in the interstitial tissue about the degenerated distal convoluted tubules and arterioles. The liver showed various degrees of damage from cloudy swelling and vascular congestion to focal or massive necrosis and infiltration of the portal areas with neutrophils and lymphocytes. The urine from animals in shock frequently contained large quantities of erythrocytic, hyaline, and granular casts. Certain unpublished data suggested to them the release of proteolytic enzyme in extensive tissue damage producing changes at the site of injury and other catabolic effects.

A report by Miller (1940) shows that protein breakdown occurs during anaphylactic shock in the dog. Many other forms of injury also produce similar effects. Yet, according to Miller, the results support the proteolytic theory of antigen-antibody combination. The severity of the clinical response to an intravenous shock dose of horse serum in a previously sensitized normal dog is definitely paralleled by the increase in urinary nitrogen. When the clinical response is severe as indicated by a profound fall in blood pressure, prolonged bloody diarrhea, vomiting, and collapse, there is a very pronounced increase in total urinary nitrogen with increase in the urea and ammonia fractions, while the creatine shows little change. These changes which have also been observed in other injuries, indicates cellular injury resulting from antigen-antibody combination.

A large increase in urinary creatine is associated with tissue injury of any type, disintegration of muscular tissue, or in fever; it occurs in the urine from carcinoma of the liver, in pregnancy, or after fasting, or intake of a high carbohydrate diet associated with muscular activity. Decreased creatine excretion has been noted in decreased carbohydrate intake. The response of smooth muscle tissue involved in the early acute stage of anaphylactic reaction could very well be responsible for the increased urinary excretion of creatine.

Increased elimination of uric acid in the urine is associated with injury to the liver, resulting in the failure of the liver to oxidize uric acid to allantoin.



Liberation of histamine by the action of snake and bee venoms on egg yolk lecithin with the formation of lysolecithin which *per se* is capable of liberating histamine has been shown (Feldberg and Kel-laway, 1938). Trethewie (1939) showed that the hemolytic activity of venoms parallels their histamine liberating capacity. Rocha e Silva (1941) expressed the opinion that the whole picture of histamine liberation could be related to the proteolytic activity of venoms. Histamine could be released from the lipoprotein films of the protoplasmic structure of cells by the action of lysolecithin on the lipid component, and by the action of proteolytic enzyme on the protein component. Digestion of the protein would lead to a destruction of the normal lipoprotein structure with the consequent release of histamine (Feldberg, 1941). According to Rocha e Silva (1941), in this respect venoms and trypsin are indistinguishable. Proteolytic liberation of histamine as the cause of anaphylactic and tryptic shocks, and certain fatal symptomatologies of snake and bee venom action constitutes a central feature of the views of Rocha e Silva (1944). After brief reference to the older literature with respect to the appearance of products of enzymatic splitting of proteins in the circulating blood of sensitized animals submitted to shock, in support of this theory, he makes the following quotation from Vaughan (1913): "We hold that sensitization develops in certain body cells a new function—that of elaborating a new specific, proteoclastic ferment."

The liberation of histamine in anaphylactic shock is attributed to the activation of cellular cathepsins. Cathepsin is a proteolytic enzyme of animal tissues and belongs, according to the Bergmann scheme, to the class of proteinases consisting of trypsin, papain- $H_2S$  and cathepsin II. These require lysine and arginine in the peptide-chain such as benzoyl-l-arginine amide and benzoylglycyl-l-lysine amide for substrates (Bergmann and Fruton, 1941; Fruton, Irving and Bergmann, 1941). Rocha e Silva is inclined to believe that histamine is bound to cells forming peptide bonds with the amino acid chain of tissue proteins. This peptide bond displays a definite specificity toward proteolytic enzymes which split the amide groups from the above mentioned type of peptide chain substrates. Chymotrypsin which does not split these substrates is practically devoid of the capacity of liberating free, active histamine from cells. On the other hand, a fractionation of papain has yielded a preparation whose ability to liberate histamine



at pH 7.3 to 7.5 ran parallel to its ability of splitting benzoyl-l-arginine amide. It may also be pointed out that ficin, which is toxic to animals (Molitor, *et al.*, 1941) and produces certain pathological symptoms comparable to those produced in severe shock, is capable of splitting specifically the amide grouping in benzoyl-l-arginine amide (Bergmann and Fruton, 1941). Rocha e Silva expresses the view that most of the histamine present in living cells is bound to arginine or to lysine and can be liberated by the action of a ferment displaying the specificity of trypsin. Rocha e Silva (1944) submitted his point of view in a schematized manner, showing the possible nature of factors involved in the liberation of histamine followed by the shock of animals. The scheme perhaps presents the relationship of the various factors when proteolytic enzymes, poisons and intracellular cathepsins are responsible for shock.

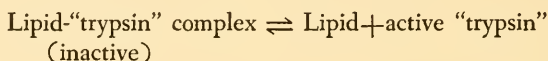
There are two types of experimental data which contradict the postulate that the liberation of histamine related to a proteolytic action. McIntire and Roth (1950) reported that histamine is released very rapidly from rabbit blood cells by two series of pure compounds (primary aliphatic amines and alkyl- $\beta$ -carbomethoxypyridinium salts). At 37°C. n-octadecylamine,  $8 \times 10^{-5}M$ , will release all or nearly all of the histamine from the normal rabbit blood cells. A maximum histamine release results also when n-hexadecyl- $\beta$ -carbomethoxypyridinium bromide is used at a concentration of  $8 \times 10^{-5}M$ . At 37°C. the histamine release by these compounds is 90% complete in one minute.

In another study, McIntire, Roth and Sproull (1950) investigated the question of whether or not histamine release from sensitized rabbit blood cells is dependent on the action of the liberated fibrinolysin as postulated by Ungar and Mist (1949). They added crystalline soybean trypsin inhibitor, which is a potent inhibitor of fibrinolysin, and purified bovine fibrinolysin and artifibrinolysin to the systems they studied. They reported that excessive concentrations of soybean trypsin inhibitor and bovine antifibrinolysin failed to inhibit the histamine release by antigen and the poor and inconsistent histamine release by bovine fibrinolysin indicated that *in vitro* anaphylactic release of histamine from rabbit blood cells does not depend upon fibrinolysin activity.

Our view concerning these problems differs from what has been discussed above. Any interpretation of the phenomenon of shock

should take into account the chain of events which occur in anaphylactic shock following the antigen-antibody reaction within or about the cells. The steps preceding anaphylactic shock must occur with tremendous speed. In anaphylaxis, the antigen-antibody reaction is claimed to occur in contact with the cells. The critical question revolves around the problem of how the combination of an antigen with an antibody fixed on tissue cells ushers in a chain of explosive reactions fatal to the host. Particularly critical is the question of how a perfectly harmless native tissue cathepsin is turned into a destructive agent by the antigen-antibody reaction. The proponents of the theory of proteolytic histamine liberation assume that cathepsin exists in an inactive state until an antigen-antibody combination takes place. In consequence of the immune reaction the activation of cathepsin is supposed to take place. The observation of Jobling and Petersen (1914) is cited to the effect that specific precipitates can activate plasma trypsin by adsorption or inactivation of plasma antitrypsin which, according to Jobling and Petersen, is a lipid, and not the serum trypsin inhibitor, a polypeptide of 6000 molecular weight. It is possible that "plasma trypsin" of Jobling and Petersen is activated by the antigen-antibody complex by removing the antitryptic lipid. This is not surprising in view of the findings of Horsfall and Goodner (1935, 1936) that lipids are part of the precipitate formed by the combination of type specific pneumococcal polysaccharide and horse and rabbit antipneumococcal serum. The removal of lipids from the antipneumococcal horse serum caused a loss of a power of agglutination and precipitation, and a marked reduction in these activities with rabbit serum. The activities of the former system could be restored by the addition of lecithin; the latter system was likewise activated by the addition of cephalin. Hilleman and Nigg (1948) reported that antigens extracted with ether from the suspensions of yolk-sacs infected with the virus of *lymphogranuloma venereum*, completely inactive in complement fixation test, were activated by the addition of: (a) alcohol soluble lipoid from the same extract; (b) alcohol soluble lipoid from normal yolk-sacs; and, (c) lecithin obtained from soybeans. The maximal reactivity of the purified antigenic fraction depended on the presence of lecithin in optimal concentration. The importance of lecithin for the activity of other complement-fixing systems has been reported. Pangborn (1942) found that purified cardio-lipin, entirely inactive in itself

in complement-fixation tests for syphilis, was activated by the addition of lecithin and cholesterol. These observations would strongly indicate that lipids exercise strong affinities for specific precipitates and thereby set free "plasma trypsin" by a dissociation reaction:



The conception of an analogy between these observations made on humoral systems and the events occurring on intact tissue cells would seem to encounter serious difficulties. Within tissue cells the synthesis of protein catalyzed by a proteinase, perhaps cathepsins, is in a dynamic equilibrium (see p. 52): Amino acids  $\rightleftharpoons$  proteins. The enzyme must therefore be constantly in an active state. To be sure, there might be inhibitors within the cellular environment. Their presence in no way, however, would seem to block the catalytic role of these enzymes in that dynamic state of protein synthesis. Most likely, the synthesis of antibody within the cells is mediated by the same enzymes supplemented with antigen. The presence of fixed antibodies in active immunization places, would, therefore, bring them within the immediate proximity or in direct contact with proteinases, probably in a complex form. It would seem, therefore, that when a new amount of antigen is reinjected into a passively sensitized or actively immunized animal, causing an anaphylactic shock, a fatal reaction takes place within or on the surface of the cells harboring the factors responsible for a series of events occurring at a speed, in severe cases, reminiscent of an electrical shock.

The combination of injected antigen with the fixed antibody, forming a complex, could attract and dislodge lipids from the lipoproteins of the protoplasm and combine with it. Such a course of events can readily cause injury to and disorganization of the cells. As will be discussed below, the mobilization of choline and synthesis of acetylcholine in anaphylaxis may be associated with an effect on the phospholipids of the lecithin type. Cellular metabolism deviating from its normal course can thus cause various other intensified catabolic reactions which have already been considered above. There are numerous observations that in damaged cells proteolysis and gluconeogenesis become predominant metabolic reactions. Injured cells would correspond to an *in vitro* reaction environment where proteinases

exercise predominantly a proteolytic function. If we assume that a proteolytic action precedes, or is necessary, for anaphylactic reactions, the antibody synthesizing proteinase might readily assume a proteolytic function in an injured tissue. Or in non-antibody producing cells local proteinases can assume such a role. Our concept, in contrast to that of Rocha e Silva, does not provide a preliminary reaction to activate cellular cathepsins. They are there in active form to regulate the synthesis and proteolysis of proteins. Under injurious conditions cathepsins simply assume predominantly a proteolytic role. In this way, the disturbed equilibrium of reactions in damaged tissues can lead to proteolysis and thus account for the increased non-protein nitrogen in the humoral system in various pathological conditions, and an increase non-protein nitrogen excretion in the urine of animals subjected to anaphylactic shocks. In this connection reference needs to be made to the results of a study by Ungar (1947). He reported that the addition of specific antigen to tissues—lung, liver, kidney—of sensitized guinea pigs causes the liberation of a protease. The same results were obtained by adding peptone to normal guinea pig tissues. Only minute amounts of enzyme were detected in the experimental conditions used in this study. However, Ungar and Mist (1949) found that fibrinolysin was liberated by adding the specific antigen to serum from sensitized guinea pigs.

## 11. Summary

An attempt to formulate a reasonable concept concerning the mechanism of anaphylaxis necessitates a recapitulation of the pertinent reactions. The anaphylactic reaction is violent and explosive in character. It is associated with profound disturbances in the respiratory, circulatory, glandular and smooth muscle systems. The antigen-antibody reaction, occurring in a cellular environment, causes injury to cells. In severe cases, it is fatal; in mild cases it may be reversible. These manifestations result in, or are associated with abnormal metabolic changes.

In anaphylaxis, there is first quickening of respiration, followed by stenosis of the bronchioles due to direct action on the muscular walls of the bronchi, leading to asphyxia. An initial moderate rise in systemic blood pressure, most likely due to the stimulation of the sympathetic



system, is followed by gradual fall in blood pressure (parasympathetic response) due to peripheral vasomotor paralysis. There is dyspnea, vomiting, salivation, diarrhea, etc. which indicate excessive stimulation of glandular secretions. Agonal convulsion is characteristic of anaphylaxis, arising, possibly, from severe anoxia or lack of oxygen supply, heart block, hypoglycemia, increased intracranial pressure, etc.

The shock reactions caused by antigen-antibody combinations, histamine, acetylcholine, peptone-like substances, certain proteinases, and trauma possess more or less close resemblances. It would seem that interference or blocking of *pulmonary*, *circulatory* and *glandular* functions of the organism may be the principal reactions occurring in shock. Other manifestations in shock associated with or resulting from them may appear to be byproducts which no doubt can intensify the course of events and precipitate fatal shock.

The specific factors which have been claimed to be responsible for the various reactions occurring in shock are *histamine*, *acetylcholine* and *tissue proteolytic enzymes*. These are individually discussed. At present the specific data are inadequate to consider specifically any one of these three factors as *the* one solely responsible for the phenomena of shock. It may be that all these factors are jointly involved.

In this connection it may be worth while to point out that while the role of proteinases in the living cell is well known, the specific nature of their activity in a cell injured by an antigen-antibody reaction is not known. In our opinion, in such a cell proteolytic enzymes could function principally as catabolic agents. In other words, the hydrolytic role of proteinases may supersede those of their synthetic functions. In an *in vitro* environment proteinases exercise on proteins principally a hydrolytic function. An injured cell in this respect may be compared to an *in vitro* environment. This may appear plausible in view of the fact that an injured cell, as in a shock, would be cut off from the flow of energy required for synthetic processes.

On comparing the anaphylactic reactions provoked by histamine and acetylcholine, we see a very close resemblance between the two in these respects. However, while acetylcholine plays an indispensable role in the normal function of the autonomic nervous system and those of voluntary muscles, and the mechanism of its action is characterized, there is as yet no information concerning the normal function, if any, or the specific receptor site for histamine action.



In considering acetylcholine as a factor in anaphylaxis, it is true that the nerve cells and heart muscle contain cholinesterase which destroys acetylcholine. But there are regular intervals during which it produces its effect. Apparently, this function is multiplied manifoldly as a result of an abnormal degree of stimulation causing inhibitions of normal functions. The specific role of histamine is likewise beset with complications in view of the fact that it can be destroyed by specific enzymes at the site of its liberation.

The data concerning anaphylaxis which have been presented in detail may perhaps lend themselves to systematization in the following sequence:

The interaction of antigen and antibody at the surfaces of susceptible cells may cause displacement of lipid or protein components of the cell surface membrane in such a way as to alter permeability relations and otherwise disturb existing kinetic and chemical equilibria within the cells. Such disturbances may initiate predominantly catabolic action by proteolytic or other intracellular enzymes resulting in liberation possibly primarily of acetylcholine and perhaps secondarily of histamine. Abnormal concentrations of acetylcholine and histamine in the circulation cause contraction of smooth muscle in the respiratory and vascular systems, promote glandular secretion and injure capillary endothelium, as well as produce their characteristic effects through the sympathetic and parasympathetic nervous systems. The impairment of respiration and of oxidation thus brought about further increases capillary permeability and loss of fluid from the circulation into the tissue spaces. The resultant anoxia further aggravates the original cellular injury.



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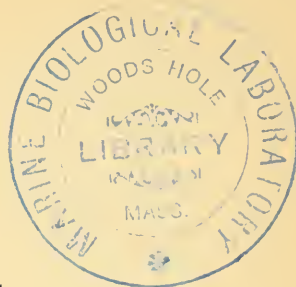
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